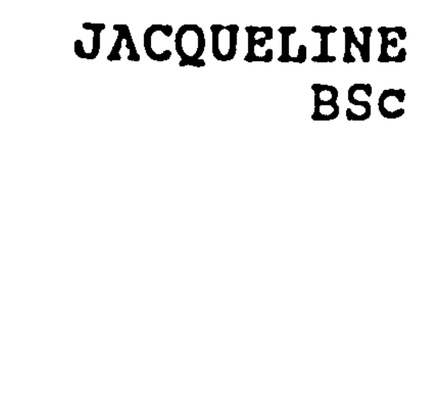
THE CULTURE, EPIDEMIOLOGY AND VIRULENCE FACTORS OF CLOSTRIDIUM DIFFICILE

by

JACQUELINE SHARP
BSc



Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1987

for

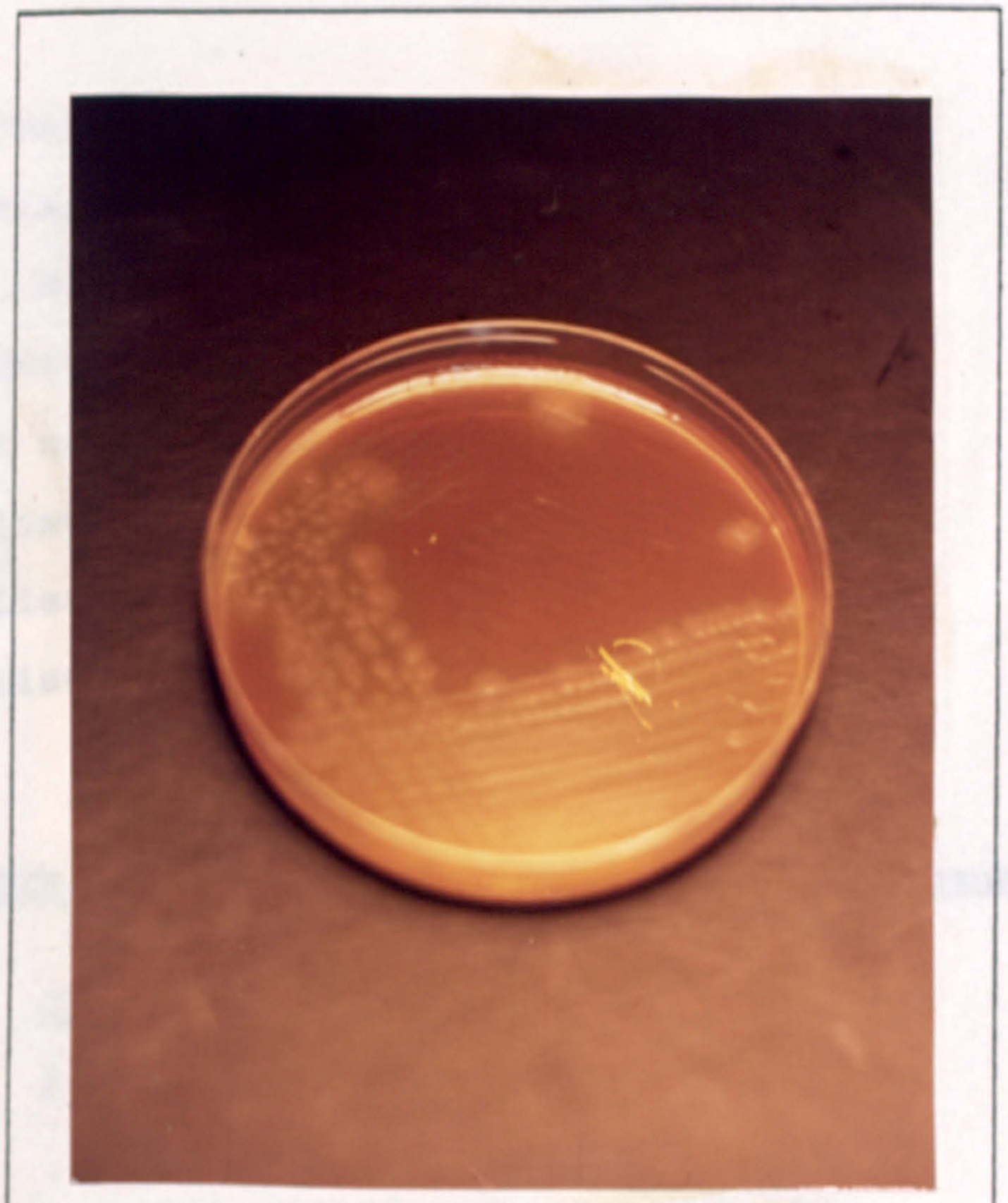
Mum

Gran and Papa

(& Dad)

'Curiosity is one of the permanent and certain characteristics of a vigorous intellect'

Samuel Johnston



CLOSTRIDIUM DIFFICILE COLONIES GROWING ON CCFA MEDIUM FOLLOWING ANAEROBIC INCUBATION FOR 48 HOURS.

TABLE OF CONTENTS

ABSTRACT

The literature on the culture, epidemiology and possible virulence factors of Clostridium difficile is reviewed with reference to the association of the organism with pseudomembranous colitis and other bowel disorders.

Studies were done to assess the use of enrichment broths for increasing isolation of C. difficile from faeces. Enrichment culture to determine the level of carriage of the organism in healthy adults resulted in isolation of the organism from 11% of such individuals. No correlation was found with previous antimicrobial therapy or the sex of individuals.

Recommendations are made for routine culture of the organism in diagnostic laboratories. It is suggested that about 1g of faeces be mixed with 1ml of industrial methylated spirits, left on the bench at room temperature for one to two minutes and then plated onto CCFA medium containing 250µg/ml cycloserine and 8µg/ml cefoxitin. Plates should be incubated for 48h.

SDS-PAGE and immunoblotting were used to characterize isolates from various outbreaks of C. difficile-associated disease where cross infection was suspected. Although cross infection was shown to be a possible mechanism of transmission in some cases, it was not the only factor involved in developing disease.

Cell surface components were extracted from strains of C.

difficile for immunochemical analysis. Two antigens were extracted from crude cell membranes. The lipoteichoic acid (LTA) moiety was shown to form a ladder pattern, reminiscent of that seen with the lipopolysaccharide from smooth Gram-negative organisms, when analysed by SDS-PAGE. This was a common antigen in four strains studied. Similar molecules were not demonstrated in *C. sordelli* or *C. bifermentans*. The identity of the second antigen is uncertain. It may be a deacylated form of the LTA-type molecule. Cell wall carbohydrate antigen, extracted from three strains, was shown to cross-react with some, but not all, heterologous antisera.

Cell wall proteins, extracted with 6*M* urea, were found to be different in each of five *C. difficile* strains studied. This indicates greater diversity in such proteins than previously described. Each strain had one to three major proteins, with molecular masses (M_r) of between 28.3kDa and 54kDa. These were always antigenic when probed with homologous antiserum (by immunoblotting) but were not detected with heterologous antisera. There was one antigen with M_r of about 73kDa that was common to all strains.

Most strains of the organism tested were found to be relatively hydrophilic in nature. However it was demonstrated that different culture techniques could alter the results obtained.

Flagella isolated from two *C. difficile* strains were

shown to have an M_r of about 38kDa. They were antigenic, showing cross-reaction with antiserum raised against different strains of the organism.

Studies were performed to assess the ability of the organism to adhere to mouse ileum. It appeared that viable C. difficile cells associated with this tissue and that the association increased with time. Bacteroides fragilis was also shown to associate with the ileum but not in the same manner as C. difficile.

Acknowledgements

I should very much like to thank my supervisor, Dr. Ian R. Poxton, for his help and encouragement throughout the past three years and in the final preparation of this thesis.

Thanks must also go to Mr. Robert Brown, Chief MLSO, for his untiring assistance and advice in all things technical.

I am grateful to the staff of the Medical Illustration Service, in particular Mr. David Dirom who has helped me on countless occasions with 'thesis jobs'. Thanks are also due to Mr. Derek Notman for his help with electron microscopy.

I must not forget the staff of Clinical Bacteriology who participated in the cultural studies done during this work and also allowed me to use their anaerobic cabinet on occasion! Mr. Alan Smith of the Pathology Department is also due thanks for his help with tissue sectioning and staining.

Finally, I would like to thank Professor J.G. Collee for his advice and encouragement throughout my time within this department. I also acknowledge the receipt of Grant K/MRS/50/C627 from the Scottish Home and Health Department which made this work possible.

LIST OF PUBLICATIONS

The results of work presented in this thesis have already been published. The references are as given below:

1. SHARP,J. and POXTON,I.R. (1985). An immunochemical method for fingerprinting Clostridium difficile. Journal of Immunological Methods, 83, 241-248.

2. SHARP,J. and POXTON,I.R. (1986). Analysis of the membrane lipocarbohydrate antigen of Clostridium difficile by polyacrylamide gel electrophoresis and immunoblotting. FEMS Microbiology Letters, 34, 97-100.

3. CUMMING,A.D., THOMPSON,B.J., SHARP,J., POXTON,I.R. and FRASER,A.G. (1986). Diarrhoea due to Clostridium difficile associated with antibiotic treatment in patients receiving dialysis: the role of cross infection. British Medical Journal, 292, 238-239.

4. SHARP,J. (in press). Immunochemistry of Clostridium difficile. In Anaerobes Today. Eds J.M.Hardie and S.P.Borriello. John Wiley and Sons Ltd, Chichester, England.

The investigations and procedures described in this thesis were performed by the author unless indicated otherwise in the acknowledgements.

A note on C. difficile toxin(s):

The early literature regarding toxin production by isolates of C. difficile has now been shown to be very much oversimplified. Initially, it was believed that strains of the organism produced only one toxin which was cytotoxic and as such could easily be detected by tissue culture assay. Thus, 'the cytotoxin' was referred to and any organism producing a cytopathic effect was described as 'cytotoxic'.

It has now, of course, been shown that C. difficile can produce other toxic factors which may also be cytotoxic. Readers of this thesis should bear in mind that references to 'cytotoxicity' and 'cytotoxic effects' in Chapters 1 to 4 will be made to studies where, in all probability, the toxin preparations tested would have been crude mixtures of several different toxic extracellular products. Chapters 5 and 6 include information about two of these toxic products, toxins A and B, which have now been studied in greater detail.

LIST OF ABBREVIATIONS

AA-PMC	antibiotic-associated pseudomembranous colitis
AAC	antibiotic-associated colitis
AAD	antibiotic-associated diarrhoea
BA	blood agar
BBA	Brucella-based blood agar
BHI	Brain Heart Infusion medium
CAPD	continuous ambulatory peritoneal dialysis
CAS	casaminoacid medium
CCFA	cycloserine, cefoxitin, fructose agar
CCFB	cycloserine, cefoxitin, fructose broth
CCFT	cycloserine, cefoxitin, fructose broth with 0.1% sodium taurocholate
CCIE	counter-current immunoelectrophoresis
CCMB	cycloserine, cefoxitin, mannitol broth
CIBD	chronic inflammatory bowel disease
CIE	crossed immunoelectrophoresis
CMB	cooked meat broth
cu	cytopathic units
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FRIE	fused-rocket immunoelectrophoresis
GI	gastrointestinal
GLC	gas-liquid chromatography
HD	haemodialysis
HRP	Horseradish peroxidase
IF	immunofluorescent

ABBREVIATIONS continued:

KR	Kreb's-Ringer solution
LA	latex agglutination
LTA	lipoteichoic acid
MIC	minimum inhibitory concentration
MPRL	Microbial Pathogenicity Research Laboratory
M_r	molecular mass
MRU	Medical Renal Unit
NB	nutrient broth
NCTC	National Collection of Type Cultures
NEC	necrotizing enterocolitis
NVFA	non-volatile fatty acid
OD	optical density
OWL	outer wall layer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMC	pseudomembranous colitis
PPY	Proteose Peptone Yeast Extract medium
p-r	pre-reduced
RCM	Reinforced Clostridial Medium
RI	refractive index
RIE	rocket immunoelectrophoresis
RIE	Royal Infirmary of Edinburgh
RT	room temperature
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCA	tissue culture assay

ABBREVIATIONS continued:

TCCFA	cycloserine, cefoxitin, fructose agar with 0.1% sodium taurocholate
TCM	tissue culture medium
u-v	ultra-violet
VFA	volatile fatty acid

List of Figures

List of Tables

CHAPTER 1

CLOSTRIDIUM DIFFICILE AND ITS ASSOCIATION WITH BOWEL DISEASE

1.1. Clostridium difficile

This organism was first isolated by Hall and O'Toole (1935) during an investigation into colonization of the human gut by bacterial species over the first few days of life. Initially, due to difficulties encountered in its isolation and subsequent study, the organism was named Bacillus difficilis. Although culture extracts were found to be extremely toxic to guinea-pigs and hamsters when injected subcutaneously, the organisms' potential for causing disease in humans was not fully recognized until its association with pseudomembranous colitis (PMC) in 1978.

1.1.1. Morphology

The organism is a Gram-positive, obligately anaerobic rod shaped bacterium. It produces subterminal oval shaped spores which become terminal given the right growth conditions. The dimensions of the organism vary (usually 1.3-1.6µm by 3.1-6.4µm) although the rod length can be increased considerably when the organism is cultured on media containing strong reducing sugars. It has peritrichous flagella and characteristic oscillatory motility in cooked meat broth (CMB).

1.1.2. Biochemistry

Studies show that biochemical reactions can vary markedly between strains of C. difficile. Most will ferment

glucose, fructose, xylose, salicin, mannitol and hydrolyse aesculin but fail to attack galactose, lactose, sucrose, raffinose, inulin and glycerol. Some produce gelatinase and all are indole and lecithinase negative (Hall and O'Toole, 1935; Hafiz and Oakley, 1976; Poxton, 1982).

1.1.3. Extracellular products

C. difficile is known to produce at least four toxins which may be involved in the pathology of *C. difficile*-associated disease (Mitchell *et al.*, 1986). Toxin A (enterotoxin) causes fluid accumulation in animal ileal loop assays. Toxin B (cytotoxin) is responsible for characteristic rounding and separation of cell monolayers in tissue culture. A further toxin (toxin C) which increases gut motility, has been described (Justus *et al.*, 1982). There is also a labile toxin causing clear fluid accumulation in rabbit ileal loops which appears distinct from toxin A (Banno *et al.*, 1984). *C. difficile* also produces a factor that inhibits neutrophil migration and phagocytosis (Bolton *et al.*, 1985).

1.1.4. Detection of C. difficile in faeces (excluding cultural methods)

Most of the methods available for identification of *C. difficile* within faeces are based on detection of the toxins (A and/or B). Since the cytotoxic activity of the organism was first described (Larson *et al.*, 1977) tissue

culture assays (TCA) have been used to establish whether or not the organism is present within clinical samples (Bartlett, 1979). However, the poor availability of cell lines and also the expertise required to maintain them has tended to limit the diagnostic usefulness of TCA. More recently commercial TCA kits, which require no special expertise, have been developed which seem to perform satisfactorily (Wu and Gersch, 1986; Nachamkin et al., 1986; Welch et al., 1985). Whichever technique is used results take up to 48h to obtain, thereby making a rapid clinical diagnosis by these methods impossible.

Various immunological techniques have been used in trying to detect C. difficile or its toxins within specimens. These include immunofluorescent (IF) labelling, counter-current immunoelectrophoresis (CCIE), crossed immunoelectrophoresis (CIE), enzyme-linked immunosorbent assays (ELISA) and latex agglutination (LA) tests.

Wilson et al. (1982b) tried to use an IF label to detect the organism but found extensive cross-reaction with other clostridial species commonly found in faeces (i.e. C. sordelli, C. bifermentans, C. chauvoei and C. sporogenes). Attempts to absorb out the cross-reactive antigens resulted in loss of reactivity against 18 of the 20 C. difficile strains being tested. Hubert et al. (1981) encountered similar problems with this technique. CCIE (in which faecal filtrates are placed in the cathodic well and antiserum in the anodic well of a gel)

and CIE (where the antigen(s) are electrophoresed in two different dimensions with antiserum incorporated into the second dimension gel) have been widely used as methods for toxin detection (Ryan et al., 1980; Welch et al., 1980; Levine et al., 1982; Wu and Fung, 1983; Jarvis et al., 1983; Rennie et al., 1984; Lyerly et al., 1985). LA (Shahrabadi et al., 1984; Lyerly et al., 1985) and ELISA (Yolken et al., 1981; Lyerly et al., 1983; Laughon et al., 1984; Redmond et al., 1985; Krishnan, 1986) have been investigated more recently.

ELISA appears to be the most sensitive technique with detection of toxin A at levels of between 0.1 and 1ng per well (Laughon et al., 1984; Lyerly et al., 1983). This is equivalent to detection of about 1 to 5ng of toxin per millilitre of faecal suspension. Most laboratories have facilities to perform ELISA and results are available in about 4h.

The major problem with these techniques at the present time is that highly specific, commercially produced antisera are not available. Most published results (obtained with CIE, CCIE or ELISA) show lack of specificity since the antisera used have been produced against incompletely purified toxins of either C. difficile or C. sordelli and all contain antibodies to many other antigens (Poxton and Byrne, 1981a; West and Wilkins, 1982; Sands et al., 1983; Laughon et al., 1984). Ryan et al. (1983) have reported increased specificity of

CCIE by use of absorbed sera and the specificity of ELISA has been improved in the experimental situation with monoclonal, affinity purified and monospecific antibodies (Lyerly et al., 1985) or absorbed sera (Krishnan, 1986). Lyerly et al. (1985) noted that monoclonal antibodies could be prepared in unlimited quantities and that they were more reproducible agents than the other preparations. However, they did appear to have decreased sensitivity in comparative studies.

Rautenberg et al. (1986) showed that immunoblotting of cell culture supernates of C. difficile against monospecific antisera could be used to detect toxin A. They found that the denatured and reduced antigen retained enough antigenicity for detection even after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). It has since been shown (Rautenberg et al. (1987) that the antibodies used in this assay do cross-react with other C. difficile proteins as well as with proteins from C. bifermentans, C. botulinum and C. sporogenes. However, toxin A was easily detected among these proteins due to its unique high molecular mass (M_r) of 230kDa. No work has yet been done to evaluate the potential use of this technique for detection of the toxin in faeces although the authors suggest it may be as sensitive as other ELISA techniques. It might also turn out to be more specific than such techniques.

Initial studies with commercial LA kits for detecting

toxin A again appear to show lack of specificity (Shahrabadi et al., 1984; Kamlya et al., 1986). Lyerly and Wilkins (1986) and Peterson et al. (1986) found that the antibody used in these assays reacted with antigen also present in non-toxin-producing strains. This finding was confirmed by Borriello et al. (1987) who also showed that similar concentrations of this latex agglutinating-antigen were produced by both toxigenic and non-toxigenic strains in vivo. The antigen was shown to be totally distinct from toxins A and B and cross-reaction with C. sporogenes, C. botulinum types A, B and A/F as well as Bacteroides asaccharolyticus was demonstrated. It was concluded that the LA test was unsuitable for detection of C. difficile toxin A but that it might be used as an alternative to culture for detection of the actual organism. Results such as these would lead one to question the reliabilty of data obtained in which LA has been used as the method of detection for toxin A (Ushijima et al., 1985).

Gas-liquid chromatography (GLC), providing analysis of metabolic end products, has been studied as a possible means for rapid identification of C. difficile within clinical specimens. Early investigations (Potvliege et al. (1981) have been criticized for lack of sensitivity (Borriello, 1981) and specificity (Brooks et al., 1984a; Pepersack et al., 1983). Levett (1984a) found that there was a false-negative rate of 59% when C. difficile

culture-positive specimens were analysed by GLC. However recent work with frequency-pulsed electron capture techniques (Brooks et al., 1984b) indicate that GLC may indeed provide a useful means for direct and rapid identification of C difficile in faeces.

1.1.5. Identification of C. difficile

Conclusive identification of C. difficile isolates is usually done by GLC of the short chain carboxylic acids they produce. Although some strains produce atypical profiles, these cannot be confused with any other Clostridium species (Poxton, 1982). There are numerous reports of the successful use of this technique to identify the organism after culture on or in various media (Moore et al., 1966; Nunez-Montiel et al., 1983; Phillips and Rogers, 1981; Brooks et al., 1984b; Gianfrilli et al., 1985; Harpold et al., 1985; Navarro-Alonso et al., 1985; Brooks, 1986; Kudhair et al., 1986).

Use of commercial identification kits based on biochemical reactions have produced mixed results. Original studies with the API ZYM system (API Laboratory Products, Basingstoke, England - Al-Jumaili and Bint, 1981; Marier et al., 1984; Levett, 1985a) indicated that such a method might prove useful in identification but noted that careful standardisation of techniques was required. Appelbaum et al. (1983) reported a high percentage of misidentifications with the API 20A,

Minitek (BBL Microbiology Systems, Cockeysville, Maryland, USA) and Anaerobe-Tek (Flow Laboratories, McLean, Virginia, USA) systems. Both Levett (1985a) and Bate (1986) reported the RapID ANA (Innovative Diagnostic Systems, Decatur, Georgia, USA) system to be very good while API-AnIDENT and Minitek were less favourable.

1.2. The association of C. difficile with bowel disorders

Following the initial discovery of C. difficile there were a few reports of its recovery from various clinical specimens including abscesses, peritoneal and pleural fluids and blood cultures (Levett, 1986; George et al., 1977). However the organism only became of real medical interest once implicated in the aetiology of antibiotic-associated PMC (AA-PMC).

1.2.1. Pseudomembranous colitis

PMC has been recognized for over 100 years as a cause of occasional morbidity and mortality in patients already ill from other diseases, often following surgery (Keighley et al., 1978a; Bolton, 1982). During the early 1950's the condition became widely recognized as a serious complication of broad spectrum antibiotic use (Reiner et al., 1952; Cummins, 1961; Pearce and Dineen, 1960). Initially chloramphenicol and tetracycline were the antibiotics most frequently implicated and Staphylococcus aureus was thought to be the pathogen involved (Hale and Cosgriff, 1957). However overgrowth of S. aureus in stools from patients receiving antibiotics is not uncommon and most of these individuals have no gastrointestinal (GI) symptoms. Furthermore, investigators failed to produce PMC lesions in experimental animals inoculated with S. aureus (Dearing et al., 1960). During

the late 1970's a clear association between cytotoxigenic C. difficile and AA-PMC was established (Larson et al., 1978); nowadays the organism is isolated from nearly 100% of cases.

1.2.1a Clinical features

The clinical features of PMC are variable. It can occur at any age, although it is more common in adults and affects women more frequently than men (Bolton and Thomas, 1986). Diarrhoea is usual but not invariable, the stool watery but rarely bloody. Onset is frequently abrupt with vomiting in the early stages. Many patients complain of cramping hypogastric pain and the physical signs can simulate acute peritonitis. Other features include pyrexia, neutrophilia, hypoalbuminaemia. The natural history of the disease ranges from mild self limiting illness to a rapidly fulminating fatal condition often involving secondary complications (Mogg et al., 1979).

Diagnosis rests on the demonstration of colonic pseudomembranes either by sigmoidoscopy or tissue biopsy. These appear as discrete yellow plaques up to 2mm in diameter, scattered over the mucosa. In severe cases the plaques coalesce. Histologically they consist of mucous debris, inflammatory cells and exudate overlying groups of partially disrupted glands.

Disease symptoms often start four to 10 days after

beginning antimicrobial treatment although onset is delayed for up to 10 weeks after completion of treatment in 25 to 40% of patients (Tedesco, 1982) and can occur after single dose antibiotic administration. Most patients with diarrhoea starting during antimicrobial administration will promptly improve if the antibiotic is discontinued (Tedesco, 1982). Prolonged diarrhoea with persistent systemic complaints is more frequent when symptoms begin after discontinuation of antibiotics or in patients continuing to take the implicated antibiotic despite diarrhoea (Bartlett, 1979).

1.2.1b Antibiotic involvement

Nearly all therapeutic agents with antibacterial activity have been implicated in the aetiology of PMC. The agents most often associated are clindamycin and ampicillin (Bartlett, 1981a; Toffler et al., 1978; Silva et al., 1984; Ambrose et al., 1985). Tedesco et al. (1974) found 10% of clindamycin recipients had PMC but other reports gave much lower figures of between 0.01% (Ramirez-Ronda, 1974) and 2% (Gurwith et al., 1977). Changing prescribing patterns most probably account for the decreasing number of cases attributed to clindamycin nowadays (Tedesco, 1984). The cephalosporins, now used with increasing frequency, have shown a corresponding rise and now account for some 30% of PMC cases (Hutcheon et al., 1978; Bartlett, 1981b; Tan et al., 1979; Tedesco, 1984).

Amoxycillin and ampicillin together account for about a further 30% of cases. In a study of 329 patients Bartlett (1981b) found clindamycin, ampicillin and cephalosporins to be associated with about 80% of diarrhoeas (not necessarily PMC cases) in which a single antibacterial agent had been administered prior to onset of symptoms. Ambrose et al. (1985) recovered C. difficile from the faeces of 31% of volunteers who had been given a single intravenous dose of a cephalosporin. Seven such antibiotics were tested and the organism was recovered from individuals receiving each of these. None of these individuals developed PMC but 53% of them did have diarrhoea. These effects were not repeated when the study was performed with various penicillins.

Vancomycin has not been associated with PMC and is in fact the first choice agent for treating the condition (Bolton, 1982). Metronidazole, which can also be used therapeutically, has been implicated in causing PMC (Thomson et al., 1981) but this should not preclude its use in therapy especially in cases of PMC relapse after initial vancomycin treatment (Bolton, 1982). PMC is extremely rare after erythromycin and parenterally administered aminoglycosides. Chloramphenicol and tetracycline, originally the most frequently implicated agents, are now rarely involved. This could be due to declining use of chloramphenical but other factors must also be involved since tetracyclines are still used with

great frequency (Finkel, 1978; Bartlett, 1981b; Collee and Amyes, 1986).

1.2.1c C. difficile and AA-PMC

In 1977, what was believed to be a specific heat-labile toxin with characteristic tissue culture cytotoxicity, was detected in the faeces of a 12-year-old girl with AA-PMC (Larson et al., 1977). Based on experiments with hamsters it was suggested that this toxin was clostridial in origin (Bartlett et al., 1977). It was found that it could be neutralized by C. sordelli antitoxin (Rifkin et al., 1978) although C. sordelli was never isolated from PMC cases. C. difficile was eventually cultured from hamsters with experimentally-induced antibiotic colitis (typhlitis) and this was soon followed by reports of the organism being isolated from human patients with PMC (Bartlett et al., 1978a; George et al., 1978a; Larson et al., 1978; George et al., 1979b). In 1978 the cumulative published experience of three laboratories showed C. difficile had been recovered from 32 of 35 patients with AA-PMC (91%) and that nearly all these strains were cytotoxigenic (Bartlett, 1979). Nowadays C. difficile is isolated from AA-PMC patients worldwide and the organism is recognized as the primary pathogen in this condition.

1.2.2. Antibiotic-associated colitis (AAC) and diarrhoea (AAD)

Diarrhoea frequently develops following antibiotic treatment (Lishman et al., 1981a), occurring with a wide range of severity (Viteri et al., 1974). However the role of C. difficile in AAC and AAD remains uncertain despite many investigations (George et al., 1979c; Tvede and Willumsen, 1982; Drapkin et al., 1985; Gerding et al., 1986). As with PMC the organism has been isolated from individuals with colitis following only one dose of an antibiotic (McNeely et al., 1985).

Initial studies suggested that about 6 to 15% of faeces from patients with AAD gave TCA results indicative of the presence of C. difficile. (Bartlett et al., 1978c; Keighley et al., 1978a). C. difficile was cultured from these specimens (Bartlett et al., 1978c) and also from 16 to 20% of specimens having no detectable cytotoxic effect. Later studies employing CCFA medium (George et al., 1979d) have shown that the organism can be isolated from about 40 to 50% of cases of AAD (Falsen et al., 1980; Brettle et al., 1982) with or without the presence of faecal cytotoxicity (Lashner et al., 1985). Falsen et al. (1980) found that C. difficile was the second commonest enteropathogenic isolate (after Salmonella) in a survey of unselected faecal specimens in a diagnostic laboratory. The organism was cultured from 33% of 168 patients from whom an enteropathogen was actually

identified.

Milder cases of AAD tend to have a lower incidence of demonstrable cytotoxicity but both C. difficile and positive TCA results are found to a greater extent in a larger proportion of cases involving more severe forms of AAC (Bolton, 1982). In such cases elimination of the measurable cytotoxicity by vancomycin (Keighley et al., 1978b) or metronidazole (Pashby et al., 1979) results in rapid clinical and sigmoidoscopic improvement. It has been suggested that the lower cytotoxicity levels detected in less severe disease may be due to the presence of fewer organisms in the gut or the presence of organisms that are inefficient toxin producers (Bolton, 1982). It may also be that the raised serum antibody levels to C. difficile toxin(s) that have been detected in some patients with AAD and PMC, may play a role in modifying the clinical syndrome seen in some individuals (Aronsson et al., 1983). This is discussed further in section 5.1.5.

Oncology patients have a high incidence of GI upsets (Malamou-Ladas et al., 1983) which are often associated with cytotoxigenic C. difficile (Morris et al., 1984). Kim et al. (1981) found up to 40% of such patients harboured C. difficile and suggested these individuals might form a highly susceptible group due to extensive antibiotic treatment regimes. C. difficile has also been recovered from children receiving antibiotics for gut

decontamination prior to bone marrow transplants and in those receiving chemotherapy for acute leukaemia (Rogers et al, 1981; Panichi et al., 1985; Rampling et al., 1985). Renal patients are another immunocompromised group from whom the organism has been isolated during GI upsets (Ritchie et al., 1982; Gokal et al., 1982; Leung et al., 1985). The great majority of these isolates have been cultured from patients receiving antibacterial agents for a range of infections.

Patients with cystic fibrosis are an interesting group since C. difficile is often isolated from them despite a lack of GI symptoms (Wu et al., 1983; Welkon et al., 1985; Peach et al., 1986). The organism, which may or may not be cytotoxigenic, appears to be isolated only from individuals receiving antibiotics at the time of isolation.

1.2.3. C. difficile and chronic inflammatory bowel disease (CIBD)

The organism has also been implicated in exacerbation of CIBD. Most studies show C. difficile and faecal cytotoxicity to be present in some patients with CIBD (Bolton et al., 1980; Keighley, 1983; Lamont and Trnka, 1980). Trnka and Lamont (1981) detected C. difficile in 19% of CIBD patients and found that cytotoxicity titres correlated with disease severity, a finding similar to results of studies into AAD. Other investigators

(Greenfield et al., 1983; Meyers et al., 1981) have found no correlation between TCA titres and disease activity. It has been suggested that the production of p-cresol from trypsin may be more important than any direct necrotic effect of C. difficile or its toxins on the gut mucosa (Editorial, Lancet, 1980). Currently no firm conclusions have been made about the role of C. difficile and/or its toxins in CIBD (Keighley, 1983).

1.2.4. Non-antibiotic-associated C. difficile disease. The association of C. difficile with bowel disorders can no longer be linked exclusively to antibiotic use. There have been a few reports of previously healthy individuals developing PMC without previous antimicrobial treatment (Howard et al., 1980; Peikin et al., 1980; Wald et al., 1980). C. difficile has been isolated from these cases and cytotoxicity was detected by Wald et al. (1980). These findings tend to suggest that, at least in some patients with non-AA-PMC, the underlying pathogenesis of the disease may be identical to that occurring with antibiotic use.

Ellis et al. (1983) reported three of 15 cases admitted to hospital with C. difficile colitis had no recent antimicrobial therapy. Other such cases of diarrhoea unrelated to antibiotic administration have also been noted (Borriello and Larson, 1981; Brettle et al., 1982; Varki and Aquino, 1982). The organism has also been

implicated in irritable bowel syndrome (Tvede and Willumsen, 1982). Ellis et al. (1983) suggested that C. difficile should be screened for in all patients with diarrhoea regardless of whether or not they have received antibacterial agents in order to prevent misdiagnosis with potentially serious consequences.

CHAPTER 2

MATERIALS AND METHODS

2.1. List of bacterial strains:

The majority of the strains used in these studies were obtained from either CMB or lyophilized culture stocks held in the Microbial Pathogenicity Research Laboratory (MPRL). All isolates sent by outside groups were classified with an MPRL number. Strains were also obtained from the National Collection of Type Cultures (NCTC) and from the Royal Infirmary of Edinburgh (RIE).

Epidemiological studies:

C. difficile	MPRL	558	(Renal Unit isolates, RIE)
	"	559	
	"	560	(see Chapter 4,
	"	589	section 4.2.5a)
	"	595	
	"	597	
	"	604	
	"	616	
	"	629	
	"	659	
	"	665	
	"	678	
	"	681	
	"	694	
	"	696	
	"	712	also MPRL 832
	"	718	" 835
	"	720	" 840 - Renal unit
	"	808	isolates used
	"	816	in section 4.2.3.
C. difficile	MPRL	1169	(Dutch isolates)
	"	1170	
	"	1171	(Chapter 4,
	"	1172	section 4.2.5b)
	"	1173	
	"	1174	Dr. E. Kuijper, Dept of Medical Microbiology University of Amsterdam.

C. difficile	MPRL 1244	(Irish isolates)
	" 1245	
	" 1246	(Chapter 4,
	" 1247	section 4.2.5c)
	" 1248	
	" 1249	from Dr. H. Humphries,
	" 1250	St. James' Hospital,
	" 1251	Dublin.
	" 1252	
	" 1253	
	" 1254	- reidentified as C.butyricum

Immunochemical studies:

C. difficile	NCTC 11223	
	MPRL 161	
	" 558	
	" 604	
	" 683	
	" 1123)	from Dr. S.P. Borriello,
	" 1128)	(see below).
C. bifermentans	NCTC 506	
C. sordelli	NCTC 8780	
C. irregularis	NCTC 11830	

Adhesion studies:

C. difficile	MPRL 1121	from Dr. S.P. Borriello, Div. Communicable Diseases, Clinical Research Centre, Harrow, Middlesex.
B. fragilis	MPRL 1282	from Dr. P.A. Garnett, Fife Area Health Laboratory, Kirkcaldy.

2.2. Isolation of C. difficile

C. difficile was isolated from faeces by culture on agar containing cycloserine, cefoxitin, fructose and egg-yolk (CCFA - George et al., 1979d - see Appendix 1 for composition). Colonies were sub-cultured onto blood agar (BA) containing Columbia Agar Base (Oxoid, Basingstoke, Hampshire) with 5% (v/v) horse or human blood. Pure isolates were stored either as Robertson's CMB cultures (Cruickshank et al., 1975) at room temperature (RT) or were lyophilised.

2.3. Culture of C. difficile

Lyophilised stock strains were reconstituted into 10ml of pre-reduced (p-r) CMB and incubated for 24h at 37°C by the standard anaerobic procedure of Collee et al. (1972) or in an anaerobic cabinet (Forma Scientific, Marietta, Ohio, USA). Isolates stored in CMB were sub-cultured to fresh p-r CMB before further use. Organisms were grown up in bulk by transferring 0.1ml of CMB culture to 100ml of p-r proteose peptone yeast extract medium (PPY - see Appendix 1 - Deacon et al., 1978), supplemented with 0.075% (w/v) L-cysteine hydrochloride and 0.04% (w/v) sodium carbonate. Actual volumes used for any particular technique were scaled up or down as required. Cultures were incubated anaerobically for 16 to 18h at 37°C.

2.4. Assessment of biochemical reactions

All fermentation patterns and biochemical characterization of strains was carried out as described by Brown et al. (in press). Tests were performed with 1ml of an overnight culture of organisms in PPY medium.

2.5. Gas-liquid chromatography (GLC)

One colony from a BA plate was cultured in PPY containing 1% glucose (Deacon et al., 1978) for 18h. To 1ml of this culture was added 0.2ml of 50% (v/v) H_2SO_4. This was centrifuged at 4,000g (20 min, RT) and the cell free supernate collected. Analysis was done on a Pye Unicam 104 gas chromatograph (Philips Analytical, Pye Unicam, Cambridge) fitted with dual flame ionization detector. A glass column (180cm x 4mm) packed with 15% SP1220, 1% H_3PO_4 on chromosorb WAW 100-120 mesh was used. Operating conditions were 147°C isothermal, with the detector at 200°C. Gas flow rates were 35ml/min for the carrier gas (oxygen-free nitrogen) and hydrogen; 525ml/min for air. For volatile fatty acids (VFA) the acidified supernate was analysed directly; for non-volatile fatty acids (NVFA), 1ml of the acidified supernate was mixed with 0.4ml concentrated H_2SO_4 plus 2ml methanol and mixed by gentle inversion (20 times) as described by Holdeman et al., 1977). After overnight incubation at RT and after gentle mixing, 0.5ml of chloroform was added. This was vortex mixed. Following separation of the layers the

chloroform layer was sampled from beneath the aqueous layer. The sample volume for both VFA and NVFA was 1µl. Identification of the metabolic end products was done by comparing peak retention times in the samples with those of standard solutions (see Appendix 1).

2.6. Cytotoxicity assay

The level of faecal cytotoxicity was assayed by observing a cytopathic effect on monolayers of either human embryonic fibroblast cells or epithelial cells in Microtitre plates (Sterilin Ltd, Teddington, Middlesex) as described by Poxton (1982). Each well was seeded with about 1 x 10^4 cells in 100µl of Eagles's (modified) Minimum Essential Medium (Flow Laboratories, Irvine, Ayrshire) containing 10% Tryptose phosphate broth, 7% calf serum, 2mM L-glutamine, penicillin (200 units/ml), streptomycin (200µg/ml) and sodium bicarbonate. The cytotoxicity of faecal supernates was assessed after emulsifying the specimen in an equal volume of phosphate buffered saline (PBS: 50mM phosphate buffer, pH 7.4, containing 0.15M NaCl) and centrifuging this at 10,000g (2 min, RT). The cytotoxicity of fresh isolates of C. difficile was estimated by assaying the supernate from five-day cultures in 3.5% (W/V) Brain Heart Infusion (BHI) medium (Oxoid). Positive titres were recorded as the highest dilution that produced 50% rounding of the cells after 24h. Incubation was at 37°C in a 5% CO_2

incubator. Neutralization was performed with 10µl of C. sordelli antitoxin (obtained from Wellcome, Temple Hill, Dartford). This was diluted 1 in 25 and added to two wells containing the first two dilutions being tested. A positive control sample was included in all tests.

2.7. Hydrophobicity assay

The procedure used was an adaptation from that described by Jessop (1986). Cultures of C. difficile grown up in PPY overnight were washed twice in PUM buffer, pH 7.1 (22.2g $K_2HPO_4.3H_2O$, 7.26g KH_2PO_4, 1.8g urea, 0.2g $MgSO_4.7H_2O$ in 1000ml distilled water). Cells were resuspended in PUM buffer to give an optical density (OD) at 470nm of about 0.3 with buffer as standard. Each suspension (3.6ml) was placed in a Wassermann Tube to which was added 300µl of octane (Sigma, Poole, Dorset). Tubes were placed in a 25°C water bath for 10 min before vortex mixing for 60 sec. Tubes were left to settle for about 30 min after which the lower aqueous phase was removed with a syringe and needle and the OD_{470} recorded again.

2.8. Preparation of antiserum

Antisera were raised against ultra-violet (u-v) - killed bacteria as described by Poxton (1979). For NCTC 11223, RIE 11831 and MPRL 161 this was in New Zealand White rabbits; for MPRL 604, MPRL 683, MPRL 1123 and MPRL 1128

Dutch rabbits were used. Bacteria were grown in 100ml volumes of PPY and washed twice in filter-sterilized PBS. The organisms were resuspended in PBS to give about 1 x 10^9 organisms per millilitre. These suspensions were irradiated as a thin film in a glass petri dish with a Bactericidal u-v unit (Hanovia Lamps, Slough, England) positioned 20cm from the cells. The irradiated cells were stored frozen (-70°C) in aliquots of 1.2ml.

When required the suspensions were thawed and 1ml given intravenously to the rabbits according to the following schedule: weeks one and two, three successive daily injections; week three, no injection; week four only one injection; week five, test bleed (5ml). All sera were tested against EDTA-extracts of the homologous strain in an ELISA to check the titres obtained. The rabbits were bled out by cardiac puncture and their sera collected.

2.9. Absorption of antiserum

This was done as described by Poxton et al. (1982). Bacteria, harvested from 50ml of an 18h culture and washed twice in PBS, were mixed concurrently with 1ml of each absorbing antiserum. The suspension was shaken during the absorption period of 30 min at 37°C on a blood-cell suspension mixer. Cells were removed by centrifugation at 5,000g for 30 min at RT. This procedure was repeated once.

2.10. Formalin treatment of cells

A 20% inoculum of a 17h PPY culture was sub-cultured into 10ml p-r PPY and incubated for 4.5h. Formaldehyde solution was added to the culture to give a final concentration of 0.2% and was incubated aerobically at 37°C for 2h. The cultures were stored at 4°C until required.

2.11. Slide agglutination - Widal test

A loopful of undiluted antiserum was placed on a clean glass slide. A loopful of the formalinized cells being tested was added to the antiserum and the two solutions mixed by gently rocking the slide backwards and forwards. A negative control was provided by mixing saline with the formalinized cells.

2.12. Enzyme-linked immunosorbent assay (ELISA)

This was adapted from Poxton (1979). Fifty microlitres of antigen diluted in 50mM carbonate buffer (pH 9.6, containing 0.02% sodium azide) to give a final protein concentration of 60µg/ml, were added to each well of a Microtitre plate (Sterilin Ltd). These were incubated at 37°C for 4h, then held at 4°C overnight. Plates were washed three times with 0.15M NaCl containing 0.05% Tween 20. Antiserum, as doubling dilutions (beginning at 1 in 400) in PBS containing 0.05% Tween and 0.02% sodium azide (antibody buffer), was added to each well (50µl) and

incubated at RT for 4h. The plates were washed as before and anti-rabbit IgG-conjugated alkaline phosphatase (ICN Biomedicals, High Wycombe, Buckinghamshire), diluted 1 in 600 in antibody buffer, was added to each well (50µl) and incubated overnight at RT. After washing the plates again, 50µl volumes of the enzyme substrate (a 1mg/ml solution of p-nitrophenylphosphate (Sigma) in 50mM carbonate buffer, pH 9.8, containing 1mM $MgCl_2$) were added to each well. After 1h incubation at RT the colour change was read on a Titertek Multiscan Spectrophotometer (Organon Teknika, St. Neots, Cambridgeshire) at a wavelength of 405nm.

2.13. Protein assay

Protein concentations were determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

2.14. Carbohydrate assay

Carbohydrates, as glucose equivalents, were assayed by the phenol sulphuric acid method of Dubois et al. (1956).

2.15. Phosphate assay

Organic and inorganic phosphate was estimated by the method of Chen et al. (1956).

2.16. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

A modified version of the method of Laemmli (1970) was used, as described by Poxton and Brown (1979). Details of the buffers are included in Appendix 2. Vertical slab gels (170mm x 140mm x 1mm) consisting of a 10mm 4% stacking gel above a 10% acrylamide separating gel were used to run up to 20 samples in a Raven Slab Gel Apparatus (Haverhill, Suffolk, England). Samples, after boiling for 3 min, were added to wells in the stacking gel and electrophoresed at 70V until they had moved through this layer (1 to 2h); voltage was subsequently increased to 150V until the sample buffer front (indicated by bromophenol blue) had travelled 7.5cm from the top of the separating gel (2 to 3h).

2.17. Staining of polyacrylamide gels

Gels containing protein extracts were stained with Coomassie blue (Poxton and Sutherland, 1976); those with carbohydrate were silver stained by a method adapted from that of Tsai and Frasch (1982). Gels were fixed in 40% ethanol (v/v) and 5% acetic acid (v/v) overnight. Oxidation was with freshly prepared 0.7% (w/v) periodic acid in 40% ethanol and 5% acetic acid for 45 to 60 min. This was followed by washing for 3 x 30 min in distilled water. Fresh ammoniacal silver nitrate solution was added (1.4ml ammmonia solution plus 21ml 0.36% NaOH with 20g

$AgNO_3$ in 100ml distilled water) for 15 min after which gels were washed in distilled water (2 x 10 min). Gels were transferred to fresh 0.005% (w/v) citric acid in 0.19% (v/v) formaldehyde and left to develop. When the required staining intensity was reached the gels were washed repeatedly in large volumes of distilled water.

2.18. Immunoblot transfer

The method of Towbin et al. (1979) as described by Poxton et al. (1984) was followed. Extracts, separated by SDS-PAGE, were transferred to nitrocellulose membrane by use of a Biorad ImmunBlot Assay kit (Biorad, Watford, Hertfordshire). Electrophoresis was at 12V (40mA, 4°C) for 18h. The membrane was washed in Tris-buffered saline (TBS: 20mM Tris, 500mM NaCl, pH 7.5) and the unbound sites were blocked with a solution of 3% gelatin in TBS for 45 min. The membrane was transferred to C. difficile antiserum (1st antibody) diluted in 1% gelatin in TBS (antibody buffer) and incubated at RT for 3h. Following two 10 min washes in 0.025% Tween 20 in TBS, the membrane was placed into goat-anti-rabbit IgG Horseradish peroxidase (HRP) conjugate (2nd antibody - Biorad or ICN Biomedicals) diluted 1 in 3000 in antibody buffer. After 1h the membrane was washed as before and placed into a solution consisting of 60mg HRP colour development reagent (BioRad), 20ml ice cold methanol, 60µl H_2O_2 and 100ml of TBS. Colour development took 5 to 20 min.

Several changes of distilled water were used to stop the reaction. All steps were performed with gentle shaking.

2.19. Extraction of whole bacterial cells with ethylenediaminetetraacetic acid (EDTA) (Standard method)

This method was as described by Poxton et al. (1984). After culture in 50ml p-r PPY, cells were harvested by centrifugation (20,000g, 5 min, 4°C) and washed twice in 50ml of PBS. The pellet was resuspended in 1ml of 10mM EDTA (BDH Chemicals, Thornliebank, Glasgow) in PBS, vortex mixed for 2 sec and placed in a 45°C water bath for 30 min. The bacterial suspensions were re-vortexed and the supernate collected after two cycles of centrifugation at 10,000g (2.5 min, RT). This was used undialysed as antigen.

2.20. Isolation of flagella

This method was adapted from Parton (1975). One litre of p-r PPY culture was harvested (13,000g, 10 min, 4°C) and washed once in an equal volume of PBS. The cells were resuspended in 20ml PBS and homogenized at full speed in a Waring blender for 2 min. The resulting suspension was centrifuged (12,000g, 10 min, 4°C), the supernate collected and re-centrifuged. This process was repeated until the supernate appeared free from any whole bacterial cells when viewed microscopically. The cleared

supernate was centrifuged at 100,000g (90 min, 4°C). Pellets obtained were resuspended in 4ml of a 0.425g/ml caesium chloride suspension in PBS. The refractive index (RI) of this was checked and adjusted with more PBS or more $CsCl_2$ to give a value of 1.3630. This is equivalent to a density of 1.3g/cm^3 (Chandler and Gulasekharam, 1974). These solutions were centifuged at 145,000g (21h, 20°C). The tubes were subsequently viewed with an inverted microscope to locate the position of any bands present. Such bands were carefully harvested from the tubes with a P1000 Gilson pipette (Anachem Ltd, Luton, Bedfordshire) after removal of overlying $CsCl_2$ solution. Each fraction collected was diluted in 10ml of distilled water and centrifuged at 100,000g (1h, 4°C). The supernate was discarded and the resulting pellet resuspended in 0.5ml of distilled water with a syringe fitted with a 19G needle.

2.21. Electron microscopy (EM) and gold-palladium shadowing

Formvar-coated copper grids were floated onto 2µl of sample. After 2 min excess moisture was removed with filter paper. The grids were placed in a desiccator to dry for at least 30 min before shadowing in an Edwards vacuum coater model 306 (Edwards, Manor Royal, Crawley, Sussex). Grids were viewed with an HU 12A Hitachi electron microscope.

2.22 Isolation of cell surface carbohydrate antigens of C. difficile

This was essentially as described by Poxton and Cartmill (1982). Bacteria from 61 of PPY culture were harvested and washed twice in PBS, pH 7.4 (10,000g, 10min, 4°C). The cells were resuspended to about 30% (w/v) in PBS and were disrupted in a pre-cooled French Pressure Cell (Aminco, Silver Springs, Maryland, USA) at a pressure of 38 to 48 MPa. DNase 1 and RNase A (Sigma) were added to the disrupted cells for 30 min before centrifuging (45,000g, 20min, 4°C) to separate membranes from walls. The supernate (containing the membranes) was frozen and lyophilised. The pellet was gently resuspended in about 15ml of distilled water and heated to 80°C for 5 min. An equal volume of boiling 5% (w/v) SDS was added and left stirring overnight at RT. The walls were washed and centrifuged at 35,000g (10 min, 20°C) with five changes of distilled water. Material was lyophilised and stored at RT.

2.23. Sodium hydroxide extraction of cell walls

The method was adapted from that of Archibald et al. (1969). The SDS-treated lyophilized cell walls were extracted with 0.5M NaOH at an approximate concentration of 0.3% (w/v) by stirring for 4h at RT. The suspension was centrifuged twice at 35,000g (30 min, 4°C) to sediment the walls. The supernate was retained and

neutralised with 1M HCl and dialysed against running water overnight, and for a further 2h against distilled water. The extract was lyophilised and stored at RT.

2.24. Phenol extraction of membranes

The lyophilised supernate was finely divided and defatted with two successive extractions (for 8h and 12h) in 200ml of chloroform/methanol (2:1 v/v). Following filtration through Whatman No.1 filter paper the residue was dried in a 37°C incubator overnight, ground to a fine powder, weighed and extracted with phenol. This was adapted from the method of Coley et al. (1975). The membranes were mixed with distilled water to give a 10% (w/v) suspension. An equal volume of 80% (w/v) aqueous phenol was added and the mixture stirred for 30 min at RT. This was centrifuged at 2,500g (20 min, 4°C) in a swing out rotor. The top layer was removed and centrifuged at 10,000g (10 min, 4°C) in a fixed angle rotor. The supernate was dialysed against running water overnight, after which an equal volume of 0.2M acetic acid/acetate buffer, pH 5.0, containing 0.02M magnesium chloride, was added. DNase 1 and RNase A (Sigma) were mixed with the extract, a drop of toluene placed on the surface of the fluid and the flask covered with a foil lid and incubated overnight at 37°C. The extracts were subsequently re-extracted with phenol as above. After overnight dialysis they were lyophilised and stored in a sealed

container at RT. This crude carbohydrate extract was further purified by gel filtration through a Sepharose 6B column (30cm x 9mm - Pharmacia, Uppsala, Sweden) with 0.2M ammonium acetate buffer, pH 6.9, containing 0.01% sodium azide. Forty fractions of about 1ml were collected. Desalting of required fractions was done on a 10ml Sephadex G25 column with distilled water.

2.25. Rocket immunoelectrophoresis (RIE), Fused-rocket immunoelectrophoresis (FRIE) and Crossed-immuno-electrophoresis (CIE)

RIE (Weeke,1973a), FRIE (Svendson, 1973) and CIE (Weeke, 1973b) were done with Shandon Southern apparatus (Camberley, Surrey). The agarose gel and buffers used are described in Appendix 2. Buffer was used undiluted in the electrode reservoirs and electrophoresis was at 60V overnight.

RIE: Antiserum (250µl), along with an equivalent volume of CIE buffer diluted 1 in 2 with distilled water and containing 2% Triton-X100 (v/v) was mixed with 3ml of agarose solution (at 52°C) and cast onto 50mm x 50mm GelBond (ICN Biomedicals - see Fig. 2.1a). Wells punched in the gel were loaded with 10µl of sample.

FRIE: 4ml agarose solution (plus 333µl CIE buffer diluted 1 in 4, with 1% (v/v) Triton X-100) was cast lengthwise onto half of a piece of GelBond measuring 100mm x 50mm. Wells punched in this were loaded with 5µl of sample. The

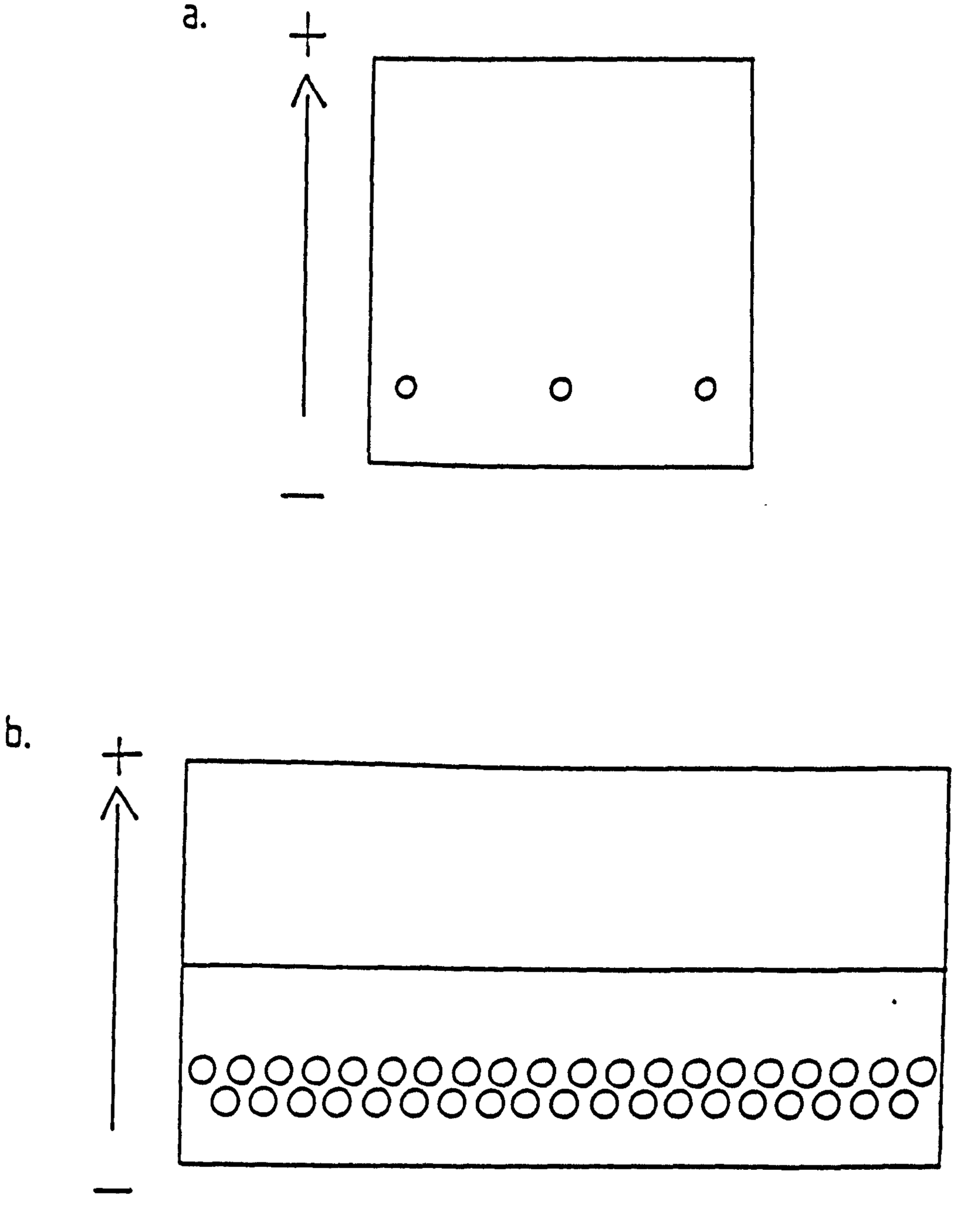


Fig. 2.1. Diagram of gels used for (a) rocket and (b) fused-rocket immunoelectrophoresis. Electrophoresis was in the direction indicated.

gel was held at 4°C for 30 min in a moist box after which 4ml agarose, containing 333µl rabbit antiserum, was cast onto the other half of the GelBond (Fig. 2.1b).

CIE: 1st dimension: Agarose (15ml) was cast onto a glass plate (80mm x 80mm). Wells punched in the gel were loaded with 12µl of sample (see Fig. 2.2a). These were electrophoresed at 60V for 1.25h at 4°C.

2nd dimension: Strips were cut from the first dimension gel in the direction of electrophoresis as shown in Fig. 2.2a. These were placed along one side of a piece of GelBond (50mm x 50mm). Agarose (3ml) containing 250µl of antiserum and 250ul of 1:2 CIE buffer, with 2% Triton X-100 (v/v), was cast onto the remainder of the GelBond. The gel was electrophoresed (60V overnight) in a direction 90° to that of the first dimension (Fig. 2.2b).

2.26. Staining of agarose gels

Following electrophoresis, gels were covered with Whatman No. 1 filter paper cut to the same size, and were pressed under 2 to 3cm of weighted blotting paper for 15 min. The paper was removed and the gels washed twice for 15 min in 0.1M NaCl, then once in distilled water. Gels were pressed once again and after removal of the filter paper were dried with a hairdryer. Staining was for at least 30 min in a solution containing 5% (w/v) Coomassie Brilliant Blue R-250; 45% (v/v) ethanol; 10% (v/v) glacial acetic acid and 45% (v/v) distilled water. The destaining

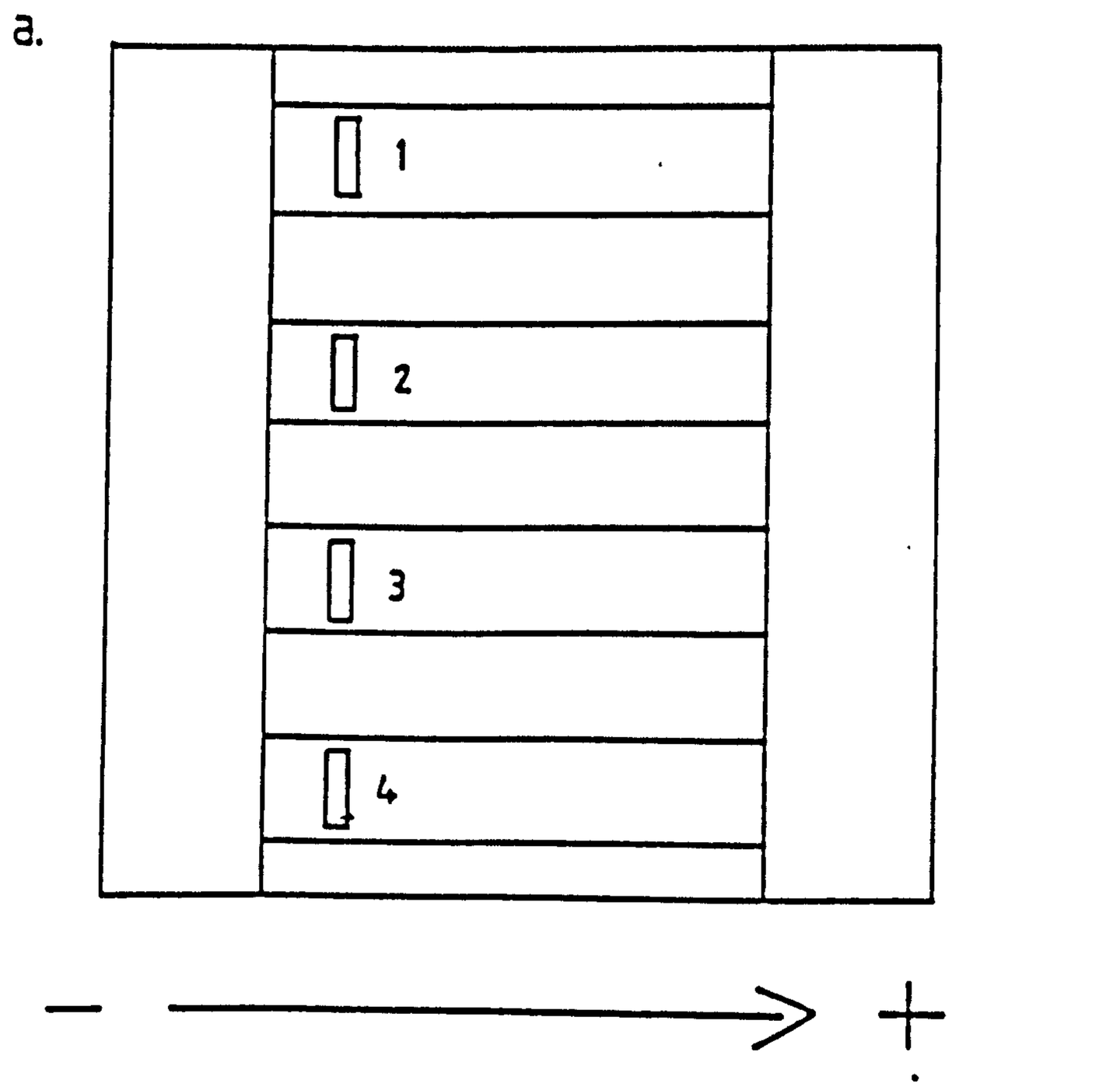


Fig. 2.2. Template used to prepare (a) first dimension gels and (b) second dimension gels for crossed immunoelectrophoresis.

solution consisted of 45% (v/v) ethanol, 45% (v/v) distilled water and 10% (v/v) glacial acetic acid.

2.27. Isolation of crude cell walls for protein extraction

Cells were cultured in 10l PPY (containing only Na_2CO_3, no cysteine hydrochloride), harvested and washed once (10,000g, 10 min, 4°C). The wash buffer consisted of 0.05M Tris/HCl, 0.025M $MgCl_2$ and 0.85% NaCl, pH 7.4. All steps were done on ice. Cells were resuspended in 40ml of buffer, DNase and RNase (Sigma - both proteinase free) were added and the cells disrupted by French pressing once, as previously described for carbohydrate extraction. After centrifugation at 20,000g (30 min, 4°C) the resulting pellet was washed four times in ice-cold distilled water (centrifuging 30,000g, 15 min). The pellet was resuspended in 10ml of distilled water and stored as 1ml fractions at -20°C.

2.28. Urea extraction of cell wall proteins

The crude cell wall material (1ml) was separated into pellet and supernate by centrifugation (50,000g, 10min, 4°C). The pellet was resuspended in 1ml of 2% Triton-X100 in distilled water (v/v) and left at RT for 30 min. Following two washes in distilled water it was resuspended in 1ml of 6M urea and again kept at RT for 30 min. Following separation into pellet and supernate

(50,000g, 10 min, 4°C) the supernate was retained and stored at -20 C.

2.29. Iodine-125 radiolabelling of cell surfaces

This was essentially as Lambert and Booth (1982). A 20% inoculum of a 16h PPY culture of C. difficile NCTC 11223 was put into 100ml p-r PPY and incubated for 3h. A total count was done with a counting chamber (Thoma, Hawksley, England) and the cells harvested and washed in PBS. Following resuspension of the cells in 1ml PBS to give about 5 x 10^9 cells/ml, 0.5ml lactoperoxidase (0.1mg/ml in PBS) was added along with 10µl of $Na^{125}I$ with an activity of 100µCi. Four 100µl portions of H_2O_2 (10mM in H_2O) were added at 2.5 min intervals. The iodination was stopped by addition of 8ml cysteine (10mM in H_2O) 10 min after the first addition of H_2O_2. The labelled cells were harvested (5,000g, 5 min), washed four times in PBS and resuspended in 0.5ml of distilled water. Extracts were stored at 4°C overnight.

2.30. Autoradiography

Iodine-125 radiolabelled sample was mixed with an equal volume of double strength sample buffer and boiled for 10 min. Samples (75µl) were applied to an SDS-PAGE gel. The separated material was transferred to nitrocellulose by immunoblotting. After washing in TBS, this was dried in a 37°C incubator and wrapped in Clingfilm. This was placed

in a Kodak X-Omatic Cl cassette (Eastman Kodak Company, Rochester, New York, USA) between two pieces of film and kept at -70°C. One was developed after 72h, the other after two weeks.

2.31. Incorporation of sulphur-35-labelled methionine into cell proteins

This was essentially as Tabaqchali et al. (1984a). About eight C. difficile colonies were sub-cultured from a 48h BA plate to 50µl of p-r PPY. ^{35}S-methionine (1µl), with about 10µCi radioactivity, was added and the suspensions incubated at 37°C for either 2h or 5h in an anaerobic jar containing GasPak (BBL Microbiology Systems, Cockeysville, Maryland, USA). When removed from the jar an equal volume of double strength sample buffer was added to each bacterial suspension. This was heated for 2 min at 100°C, cooled to RT, and the bacterial proteins subsequently separated by SDS-PAGE (40µl sample volume). The gel was sealed in a plastic bag (Pifco Vacuum Sealer), frozen to -20°C and placed in a Kodak X-Omatic Cl cassette for one week at -70°C.

2.32. Isoelectric focussing

This was as outlined in 'Isoelectric Focusing principles and Methods' (Pharmacia). Urea extracted protein samples (10µl) were applied directly to the agarose gel and were electrophoresed at 5W, 150mA and 1500V for 2h. The

volthour integrator was set at 2000. Gels were dried and stained with Coomassie blue.

2.33. Chromatofocusing

This was done as described in 'Chromatofocusing - with Polybuffer and PBE' (Pharmacia). A 30mm x 9mm column was packed with Polybuffer exchanger PBE 94 after it had been washed in 0.025M imidazole-HCl solution (Sigma, pH adjusted to 7.4 with 1M HCl after degassing). Polybuffer 74 was made up as indicated in the instructions i.e. the pH was adjusted to 4.0 with 0.5M HCl and the solution made up to 200ml with distilled water. This was not degassed. After 5ml of this buffer had flowed into the column the sample (in imidazole buffer) was applied. Fractions of about 1ml were collected. Before collection, these passed through a flow cell fitted in an SP6-550 UV/VIS spectrophotometer (Pye Unicam) set at a wavelength of 280nm. Absorbance was recorded by a Servoscribe 1S pen-recorder (Belmont Instruments, Glasgow), set at an amplitude of 10mV and a chart speed of 30mm/h.

2.34. Embedding and sectioning of mouse ileum

The tissue was placed into 10% formalinized-saline overnight. Dehydration was done by passing the tissue through a series of progressively more concentrated ethanol baths, each for 2h. The first contained 70% ethanol, the next 80%, then 90% and finally there were

three successive changes of absolute ethanol. The tissue was cleared in two changes of chloroform. Paraffin embedding was performed with three changes of 58°C melting point paraffin wax, with the third change being done in a vacuum embedder. The tissue was finally blocked out in the same wax. Sections (3 to 5µm thick) were cut with a Leitz rotary mictotome (E. Leitz Instruments, Luton) and were transferred to glass slides.

2.35. Staining of tissue sections

Sections were deparaffinized and taken to water before staining. Two techniques were used for staining, (a) the Periodic acid-Schiff (PAS) reaction and (b) Alcian Blue. Both were done as described by Cook (1972).

CHAPTER 3

CULTURE OF CLOSTRIDIUM DIFFICILE AND ISOLATION OF THE ORGANISM FROM HEALTHY INDIVIDUALS

3.1. Introduction

Clostridium difficile was first isolated from the faeces of four of 10 infants involved in an investigation of bacterial colonization in neonates. The organism grew following faecal inoculation into constricted tubes containing dextrose broth (Hall and O'Toole, 1935). During subsequent investigations, it was noted that the organism had a slow growth rate and that it was difficult to isolate in pure culture for further studies; hence its original name Bacillus difficilis.

The next report of the organism being isolated was by Snyder (1940). He used deep brain medium that had been freshly boiled and then cooled to isolate the organism from 10 of 22 babies on 28 different occasions.

3.1.1. Isolation of C. difficile from cases of AA-PMC

The first reported isolations of C. difficile from cases of AA-PMC were made with a variety of media commonly used for growth of anaerobes. During a study of patients suffering from PMC and post-operative diarrhoea George et al. (1978a) isolated clostridia on lysed BA containing either kanamycin sulphate (70µg/ml) or nalidixic acid (10µg/ml). C. difficile was isolated from each of eight patients with colitis and six of 20 patients with post-operative diarrhoea. Bartlett et al. (1978a) isolated the organism from four patients (three with

AA-PMC; one with AAD). All strains grew well on Brucella-based BA (BBA) containing menadione (10µg/ml) and clindamycin (10µg/ml). Larson et al. (1978) used 'selective or enrichment media' to isolate C. difficile but the selective agents were not specified.

3.1.2. Development of selective media for isolation of C. difficile

A selective medium is one which contains one or more substances that will inhibit growth of all but a few types of bacteria. It will facilitate isolation of particular species from a mixed inoculum. If a selective medium is agar based, quantitative analysis can be done on the level of a particular organism initially inoculated onto it.

If a liquid medium favours the multiplication of a particular species, either by containing enrichments that selectively favour it or inhibitory substances that suppress competitors, cultures from mixed inocula are termed enrichment cultures. Such cultures fail to indicate the proportion of the species present in the original inoculum.

Initial isolations of C. difficile from faeces were not made with selective media specific for the organism. Consequently, many other anaerobic bacteria grew during culture making isolation of C.difficile difficult and

time consuming. Hafiz and Oakley (1976) were the first to try and develop a selective medium specifically for growth of this organism. It was known that C. difficile could deaminate phenylalanine, first to p-hydroxy-phenylacetic acid (as do most clostridia that attack this substrate) and then decarboxylate this to p-cresol (Elsden et al., 1976). Assuming an organism that produces p-cresol must by necessity be resistant to it this appeared a useful ingredient to include in a selective medium. It was further noted that none of the other 22 clostridial species examined did this.

Hafiz and Oakley (1976) found that all the 30 C. difficile isolates used in their studies grew in Reinforced Clostridial Medium (RCM) containing 0.4% p-cresol while other clostridial species failed to grow in medium containing even 0.1% p-cresol. From their studies they concluded that RCM plus 0.2% p-cresol would provide a potentially useful selective medium.

However subsequent investigations indicated that such a medium may not always be satisfactory for isolation of C. difficile. George et al. (1979d) tried to isolate the organism on an agar containing 0.2% p-cresol but without success while Larson (1978) obtained no growth of C. difficile in RCM containing 0.2% p-cresol despite growth of the organism in plain RCM. Willey and Bartlett (1979) plated p-cresol broth cultures onto medium containing cycloserine (250µg/ml) and cefoxitin (10µg/ml) expecting

this to be highly selective for the organism but it was found that only three of 18 cultures yielded C. difficile in pure culture.

3.1.3. Use of CCFA medium for isolation of C. difficile

The major breakthrough in isolation of C. difficile from faeces came with the development of CCFA medium by George et al. (1979d - see Appendix 1 for composition). This medium exploited the resistance of C. difficile to D-cycloserine and cefoxitin and its ability to ferment fructose. The concentrations of the two selective agents were chosen based on the level of resistance of 16 isolates whose minimum inhibitory concentration (MIC) for cefoxitin was 32µg/ml and for cycloserine was 1024µg/ml. With this agar (containing 500µg/ml cycloserine and 16µg/ml cefoxitin) it was noted that the viable counts obtained were not appreciably different from those on BA.

Colony description: After 48h anaerobic incubation at 37°C colonies tend to be about 7.5mm in diameter (compared to 4.5mm on BA). They are yellow, with a ground glass appearance, circular with a slightly filamentous edge, flat to low umbonate in profile and are lipase and lecithinase negative. They also produce golden yellow fluorescence when viewed under long wave u-v light. During growth the initial orange colour of the medium often changes to yellow for 2 to 3mm around the colony.

Cells growing on CCFA have an altered morphology, usually being elongated (up to 8µm in length) by the presence of the antimicrobial agents. However, after only one subculture onto BA they revert to typical morphology. The selectivity of CCFA is such that only after 24 or 48h incubation do other colony types appear. These are usually small white or pink colonies which are circular and raised and can in no way be confused with C. difficile.

Since the initial development of this agar further studies have indicated that the concentrations of the antimicrobial agents employed can be inhibitory for some isolates of C. difficile (George et al., 1979a; Dzink and Bartlett, 1980). Levett (1985b) compared the rate and level of isolation of C. difficile from 33 faecal samples. Initial culture on CCFA containing half the usual concentration of antimicrobial agents (i.e. 250µg/ml cycloserine and 8µg/ml cefoxitin - 1/2 CCFA) resulted in recovery of C. difficile from all of these specimens. However, when re-examined by culture on full strength CCFA only 75% of the samples yielded the organism. Moreover, the degree of growth on the 1/2 CCFA plates was invariably greater. Willey and Bartlett (1979) developed an agar containing cycloserine and cefoxitin independently of George et al. (1979d). With this medium (which contained 250µg/ml cycloserine and 10µg/ml

cefoxitin) the organism was isolated from 71 of 73 stools in which C. difficile-associated cytotoxicity had previously been detected. A 67% increase in isolation of the organism was obtained when 18 stools, positive for C. difficile cytotoxicity, were examined by use of this medium compared to BBA. Phillips and Rogers (1981) used 1/2 CCFA also containing 0.1% (w/v) p-hydroxyphenylacetic acid to isolate C. difficile as they again found that full strength CCFA reduced quantitatively and qualitatively the growth of stock strains of the organism.

3.1.3a Use of further selective procedures in conjunction with CCFA

Buggy et al. (1985) compared plating of faecal specimens onto CCFA with use of such plates containing 0.1% sodium taurocholate (TCCFA). This is a primary bile salt that encourages germination of spores and has been used successfully to increase isolation of clostridia from humans. They reported that both crude and pure sodium taurocholate significantly increased recovery of C. difficile. This was in contrast to Wilson (1983) who reported that crude extracts of sodium taurocholate could inhibit growth whereas pure synthetic taurocholate or cholate enhanced growth. Buggy et al. (1983) had previously indicated that TCCFA would allow recovery of spores 20 to 25 times as great as on CCFA. Wilson et al.

(1982a) found that TCCFA quantitatively recovered vegetative forms of C. difficile in the same numbers as CCFA while recovery of spores was a mean 1.7 $\log_{10}$ higher on TCCFA than on CCFA.

Alcohol shock has been used to kill selectively vegetative forms of the bacterium, as well as other non-sporing faecal organisms, before isolation of resistant spores (Borriello and Honour, 1981; Levett, 1984b). Borriello and Honour (1981) did a comparison of direct plating onto CCFA (containing 250µg/ml cycloserine and 10µg/ml cefoxitin) with plating following treatment with ethanol at a final concentration of 50%. This study indicated that such treatment of samples could aid isolation of the organism. It was suggested that it might be especially useful in laboratories lacking the selective CCFA medium. Willey and Bartlett (1979) however found that 50% (v/v) alcohol could decrease isolation of the organism.

Heat shock is another method that has been used to selectively isolate sporing forms of the organism (Wilson et al., 1982a; Stark and Lee, 1982). Spores of C. difficile are resistant to heating at 80°C for 10 min and 70°C for 20 min (George et al., 1979d). In their study Wilson et al. (1982a) found that after such treatment the best recovery of the organism was achieved with TCCFA.

3.1.4. Use of broth cultures to recover C. difficile

Both CCFA and 1/2 CCFA plates are now used extensively for routine isolation of C. difficile. However, due to the restrictions on the size of faecal sample applied to such plates approximately 10^2 organisms per gramme of faeces must be present before the organism can be detected (Wilson et al., 1982a). In many cases the organism may be be present in lower numbers as for example, following vancomycin therapy (Onderdonk et al., 1980). Similarly low numbers may be present in specimens examined in epidemiological studies. Consequently, several groups have investigated methods for increasing recovery of low levels of the organism that might exist within samples.

3.1.4a Use of Cooked Meat Broth

Pre-reduced CMB will support growth of any anaerobes inoculated into it since it contains no selective or inhibitory ingredients. Consequently, it may not be all that useful for recovery of any organism that is present at very low levels or that does not grow as rapidly as other organisms also inoculated. Levett (1984b) used CMB to try and increase isolation of C. difficile compared with direct plating onto 1/2 CCFA. Direct plating resulted in recovery of the organism from 15 of 143 stools (10.5%) from individuals suspected of having C. difficile-associated diarrhoea. Inoculation of the

samples into CMB increased this to 33 of the specimens (23%). Both Brettle et al. (1982) and Stark et al. (1982) used CMBs during epidemiological investigations but there is no indication given as to whether the organisms isolated during the studies were obtained from direct culture or by use of these enrichments.

3.1.4b Enrichment Broths

There have been a variety of CMBs containing various antibiotics used to enrich for C. difficile. Holst et al. (1981) compared the use of direct plating onto CCFA with use of chopped meat glucose broth containing cycloserine (500µg/ml) and either kanamycin (100µg/ml) or cefoxitin (10µg/ml). No difference in the level of recovery of the organism from 117 children was found with the three different media. Buchanan (1984) devised a CMB containing increased levels of carbohydrates and also the antimicrobials cycloserine and cefoxitin at 500µg/ml and 16µg/ml respectively. It was found that there was a 16% increase in recovery of the organism after the enrichment compared to direct culture on CCFA (as detected by GLC and subculture onto BA).

It is difficult to assess which enrichment method is best, since other factors in the isolation procedure (e.g. the age of the specimens, methods of transport and storage, anaerobic techniques used etc.) also influence results. Levett and Margaritis-Bassoulis (1985) compared

three enrichment media (CMB alone or with cycloserine (250µg/ml) and cefoxitin (8µg/ml), or 0.1% sodium taurocholate). All faecal samples were subcultured onto 1/2 CCFA after five days. All three enrichment media gave higher isolation rates than direct culture. The isolation rate was marginally higher with taurocholate-containing broth than with the unselective CMB. Surprisingly, isolation rates in the antimicrobial-containing CMB were lower than in the other two broths. Chang and Gorbach (1982) used antimicrobial-containing CMB to facilitate the rapid detection of C. difficile by TCA but did not report use of the broth in isolating the organism per se. A few other studies involving non-CMB based enrichment media have been done. Carroll et al. (1983) used a BHI broth supplemented with 5µg/ml gentamicin, 250µg/ml cycloserine and 8µg/ml cefoxitin to try and isolate C. difficile. An additional seven isolations of the organism were made with this broth compared to direct plating onto 1/2 CCFA (with which 12 C. difficile had been isolated). O'Farrell et al. (1984) used a cycloserine cefoxitin fructose broth (500µg/ml and 16µg/ml respectively) containing 0.1% sodium taurocholate (CCFT) for the isolation of C. difficile from vaginal and faecal specimens. They concluded that the use of such a broth culture was a useful addition to direct plating when only low levels of the organism were likely to be present in a specimen.

It seems clear that a significant increase in the isolation rate of C. difficile may be expected when enrichment culture is performed in addition to direct culture on a selective medium. The sensitivity of a particular technique may vary widely between one study and another. This may in part be due to other factors involved in different isolation procedures as well as potential differences in the samples studied. The potential for use of enrichment techniques in epidemiological studies (especially in investigations of low level reservoirs of the organism) is an important area for further studies.

3.1.5. Isolation of C. difficile from healthy individuals

Within the healthy adult population C. difficile has been isolated from both the GI and the genital tracts.

3.1.5a Gastrointestinal carriage in adults

The majority of investigations performed in the United States and in Europe have indicated that faecal carriage of C. difficile is uncommon in healthy adults who have not received antimicrobial therapy (see Table 3.1). In these studies, C. difficile has been isolated from only 3% or less of the individuals examined. Faecal cytotoxicity has not been detected in any of them.

There are a few reports of the organism being isolated from a higher proportion of healthy individuals. Wilson et al. (1982b) cultured C. difficile from 5 of 39 (15%) normal adults with CCFA. Bender et al. (1986) found 22% of 40 elderly individuals carried the organism. Nakamura et al. (1981a) recovered the organism from 23 of 149 (15.4%) healthy young Japanese adults examined and 15 of 213 (7.0%) healthy elderly adults. The faecal count of C. difficile in these individuals was found to range from 10^2 to 10^7 organisms per gramme faeces. A cytotoxic effect (assessed by TCA of crude faecal supernates) was not detected even among those adults carrying more than 10^5 C. difficile (shown to be cytotoxigenic in vitro) per

TABLE 3.1

Some reported detections of C. difficile from healthy adults

Reference	No. of subjects	No. of specimens	C.d. +ve	Toxin +ve	Selective Medium
George *et al.* (1978b)	137	146	4	0	-[a]
Larson *et al.* (1978)	11	41	0	0	-[a]
Willey and Bartlett (1979)	-[b]	60	0	0	+[c]
Bartlett *et al.* (1980)	60	-[b]	-[d]	0	-[a]
Viscidi *et al.* (1981)	60	-[b]	0	0	+[c]
Borriello and Larson (1981)	26	62	0	0	-[a]
Varki and Aquino (1982)	21	-[b]	1	-[b]	+[e]
Greenfield *et al.* (1983)	-[b]	70	1	0	+[e]

a: selective medium not used
b: information not given
c: agar containing cycloserine 250µg/ml; cefoxitin 10µg/ml
d: results based on tissue culture assay only
e: CCFA (George *et al.*, 1979d)

gramme of faeces.

Faecal specimens from adults that produce positive TCA results are almost always associated with diarrhoea although Lishman et al. (1981b) did report cytotoxic titres of up to 10^5 cytopathic units (cu) in asymptomatic adults receiving antibiotics. Titres were more usually in the range 10^1 to 10^3 cu. In patients with PMC, cytotoxicity has been detected when the number of C. difficile has been as low as 10^2 organisms per gramme of faeces (Bartlett et al., 1978c).

In spite of the absence of C. difficile-associated cytotoxicity in the faeces of healthy Japanese adults, antibody against crude toxin preparations was demonstrated among young adults. This was at a rate of 90% among those actually carrying isolates of the organism in their faeces that were TCA-positive in vitro. Even the sera from 19% of young adults from whom C. difficile was not isolated contained such antibody. In contrast, antibody was not demonstrated among elderly adults, no matter how abundant the cytotoxigenic organisms.

Because of the variety of methods used to cultivate the organism (Nakamura et al. (1981a) used 1/2 CCFA in their studies), it is not possible to say whether the reported disparities in levels of isolation of C. difficile arise due to variations in culture techniques or genuine differences in the prevalence of GI carriage of the

organism among distinct populations. There is one report (Finegold et al., 1974) comparing the faecal flora of individuals eating either a western diet or a Japanese diet. C. difficile was isolated from one of 15 individuals on the Japanese diet; the organism was not recovered from any of the 18 persons on a western diet. However these results were obtained with too small a group of subjects to be particularly significant.

There are several other reports of attempted isolations of C. difficile from various 'healthy' populations. However many of the individuals involved in these studies are patients in hospital for some other reason apart from diarrhoea (Varki and Aquino, 1982; Brettle and Wallace, 1984) or in fact do have diarrhoea that is not associated with antimicrobial usage (Falsen et al., 1980; Borriello and Larson, 1981). Other individuals often included as 'normals' are patients receiving antimicrobials who have no GI symptoms (Viscidi et al., 1981). Since the individuals in these groups cannot be regarded as entirely healthy discussion about them is not included here. However it should be noted that C. difficile is isolated from such groups only at the very low levels associated with completely healthy individuals.

3.1.5b Genital carriage

The available evidence about genital carriage in men and women has been accumulated from studies of patients attending antenatal and postnatal clinics and departments of genitourinary medicine. O'Farrell et al. (1984), Tabaqchali et al. (1984b), and Thirkell et al. (1984) found carriage rates of 18 to 24% in antenatal patients. They reported that few specimens/swabs grew C. difficile when plated directly onto CCFA compared with enrichment culture in CCFT broth. In the study of Thirkell et al. (1984) 0.2% p-cresol broth also gave only low isolation rates. Carriage was not detected in any antenatal patients by Al-Jumaili et al. (1984) or Bolton et al. (1984) by direct culture of swabs onto CCFA or into 0.2% p-cresol broth (in the latter study). Holst et al. (1981) were also unsuccessful in recovering the organism from the vagina.

Reports relating to genitourinary clinics are also conflicting. With the use of 0.2% p-cresol broth Hafiz et al. (1975) reported carriage of the organism in 72% of 108 women. O'Farrell et al. (1984) isolated the organism from nine of 82 patients (11%) using CCFT but were unable to demonstrate the carrier state with 0.1% p-cresol broth. Thirkell et al. (1984) recovered C. difficile from six of 50 women, again with CCFT. However other workers (Moss, 1983; Levett, 1984c) have not identified any

carriers. Masfari et al. (1983) recovered no C. difficile from 28 asymptomatic controls.

Similar differences have been observed in men attending genitourinary medicine departments. Hafiz et al. (1975) isolated the organism from all of 42 men with non-specific urethritis whilst only two patients out of 150, both with balanoposthitis, were detected in studies by Masfari et al. (1983). All twenty patients investigated by Levett (1984c) were culture negative.

The major concern regarding recovery of C. difficile from healthy adults is whether or not the culture media and methods used have been sensitive enough to detect small numbers of the organism. As discussed previously many studies have been done confirming that alterations in culture methods can markedly affect the ability to detect C. difficile (George, 1986). It is clear that the use of selective broth media increases the ability to detect the organism in both faecal (Mulligan et al., 1982) and genital (Tabaqchali et al., 1984b) specimens. Also media enhancing recovery of spores can markedly increase recovery of the organism.

These findings would indicate that previous studies done to detect carriage of C. difficile that have not employed a broth medium or a selective medium designed to increase detection of spores may have underestimated the actual prevalence of C. difficile as normal flora. Thus it would

appear that the significance of endogenous carriage of C. difficile in adults is still uncertain and warrants further investigation.

3.1.5c C. difficile in healthy infants

Despite the fact that C. difficile is rarely detected in the healthy adult population and while there may be a transient rise in asymptomatic faecal carriage following antibiotic therapy (Ambrose et al., 1985), occasionally with positive cytotoxic effects, the finding of C. difficile in the faeces of an adult is usually associated with symptoms. This however is not the case with children. Faecal colonization by C. difficile, often associated with high cytotoxicity titres, has been reported on numerous occasions in normal healthy children (Collignon et al., 1986).

Chang et al. (1979) reported detection of C.difficile cytotoxicity in 2% of neonatal specimens studied. Holst et al. (1981), in a detailed study found C. difficile in only 4% of infants under one month of age. All isolates were cytotoxigenic in vitro. A more recent report (Merida et al., 1986) did not find the organism in any full term neonates but did culture it from five of 32 (16%) premature neonates. None of these strains produced a positive TCA result. These reports contrast with the majority of investigations of neonatal colonization where levels of 30 to 40% are not uncommon (Hall and O'Toole,

1935; Stark and Lee, 1982; Bolton et al., 1984; Viscidi et al., 1981). Donta and Myers (1982) suggested that there was rapid colonization of the infant gut over the first few days of life with concomitant or subsequent cytotoxicity. Despite obvious discrepancies in these reports as to the precise age of colonization with the organism there is general agreement that a high proportion of infants do carry the organism from a very early age. Counts of between 10^3 and 10^7 organisms per gramme of faeces are common (Stark et al., 1982).

Holst et al. (1981) reported colonization in 64% of infants aged one to eight months with up to 79% of those between three and five months being C. difficile-culture positive. All isolates were cytotoxigenic in vitro. Stark et al. (1982) reported 90% colonization of infants during the first year of life. Other reports suggest rates of 30 to 50% (Cooperstock et al., 1983; Richardson et al., 1983; Viscidi and Bartlett, 1981; Merida et al., 1986). Following the high rates of isolation of the organism reported during the first year of life a dramatic decrease in recovery of the organism occurs after 12 months of age with reported isolation rates approaching the low levels found in the adult population. (Holst et al., 1981; Nash et al., 1982; Stark et al., 1982).

3.1.6. Association of C. difficile with disease in children

In any investigations concerning the involvement of C. difficile in GI disease in children results are difficult to interpret due to the occurrence of the organism (apparently without causing symptoms) in many such healthy individuals. However, below is a brief summary of conditions the organism has been associated with.

3.1.6a PMC: PMC is very rare in neonates and infants although cases have been described both with (Donta et al., 1981; Richardson et al., 1981; Mandal et al., 1982) and without (Hyams et al., 1981) previous antibiotic exposure. Amoxycillin and ampicillin seem to be the most commonly implicated agents. Viscidi and Bartlett (1981) documented 10 cases of cytotoxicity positive AA-PMC in children (aged between four and 17 years) and identified in the literature a further 25 cases with 28% mortality. As in adults AA-PMC can occur in previously healthy patients receiving a short course of antibiotics for trivial infections. C. difficile does not appear to be related to AAD in children in the absence of pseudomembranes (Don and Davis, 1981; Elstner et al., 1983).

3.1.6b Infantile diarrhoea: A role for C. difficile has been proposed in some cases of infantile gastroenteritis

(Ellis et al., 1984), failure to thrive (Liston, 1983; Thompson et al., 1983), sudden infant death syndrome (Cooperstock et al., 1982) and chronic diarrhoea (Sutphen et al., 1983). However the role of C. difficile in the aetiology of any of these conditions is obscure and difficult to evaluate (Luzzi et al., 1986).

3.1.6c Necrotising enterocolitis (NEC): C. difficile has been suggested to be involved in this potentially lethal disease of sick and premature newborn infants. Although faecal cytotoxicity may be detected in some patients (Lishman et al., 1984; Cashore et al., 1981) the weight of published evidence does not support a major pathogenic role for C. difficile in NEC (Chang and Areson, 1978; Stoll et al., 1980; Sherertz and Sarubbi, 1982; Thomas et al., 1984).

3.1.6d Enterocolitis in Hirschsprung's disease: In 1982 C. difficile was incriminated in the aetiology of the enterocolitis associated with this condition (Thomas et al., 1982; Cooperstock et al., 1982) and confirmed in a subsequent prospective controlled study (Thomas et al., 1985). The major pathogenic role of C. difficile in this disease appears confined to children under three years of age.

3.1.7. Aims of this study:

1. to assess the effectiveness of enrichment broth for detecting low levels of C. difficile in faeces.

2. to try and discover if the previously low levels of isolation of C. difficile from normal healthy adults could be boosted by means of enrichment culture.

3. to provide an easy, quick and reliable method for isolation of C. difficile in faecal samples sent to the diagnostic laboratories.

3.2. Results

3.2.1. Enrichment studies

All enrichment studies were done within an anaerobic cabinet (Forma Scientific) with p-r broths and agar plates.

3.2.1a Use of enrichment cultures

It is likely that C. difficile could exist in the GI tract of normal healthy adults at levels too low to be detected by direct plating onto CCFA medium. The use of a broth containing the same selective agents as this agar medium seemed potentially useful as a means of enriching any low levels of the organism that might exist within faeces.

Assessment of CCFB and 1/2 CCFB for recovery of vegetative cells and spores in the presence of normal faecal flora

a) From vegetative cells: A 17h PPY culture of MPRL 558 (0.5ml) was inoculated into 10ml of PPY and incubated for 2.5h. A total count was made with a counting chamber (Thoma) and the culture diluted with nutrient broth (NB) to give 500 organisms/ml. Samples (20µl) were inoculated into 10 x 10ml CCFB (essentially CCFA containing 500µg/ml cycloserine and 16µg/ml cefoxitin but lacking the egg-yolk and the agar). Also, 20µl were inoculated into

10 x 10ml 1/2 CCFB (250µg/ml cycloserine and 8µg/ml cefoxitin). A 0.4ml sample of a 1 in 4 dilution of pooled C. difficile-negative faeces (as determined by previous enrichment culture in CCFB) was also added to each broth. After inoculation of each broth a further 20µl of the C. difficile suspension was put onto BA to give an indication as to the actual number of organisms each culture was receiving.

b) From spores: A loopful of CMB culture of MPRL 558 was spread onto a BA plate and incubated at 37°C for 4 days. Colonies from this plate were suspended in PBS, pH 7.4, and washed twice before heating to 70°C for 20 min to kill any vegetative cells. The spores were counted in a chamber and resuspended to give about 500/ml. These were inoculated into CCFB and 1/2 CCFB as described above. The spore suspension was also added to CCFB and 1/2 CCFB containing 0.1% sodium taurocholate (Sigma).

All broths and plates were incubated at 37°C for 48h. A loopful of broth from each was spread onto CCFA after 24, 48 and 72h to check for growth of C. difficile.

When the vegetative cells were inoculated the organism was recovered from all of the CCFB and 1/2 CCFBs after 24h incubation, each broth having received between 2 and 12 organisms. These had survived incubation with normal

faecal flora equivalent to that found in 0.1g of faeces, the usual inoculum used in enrichment work in this laboratory. When the experiment was repeated with the sporing culture C. difficile was not recovered from either of the broths until after 48h incubation. The addition of sodium taurocholate did not result in quicker recovery of the organism. It was noted in all the studies that there were many more plate contaminants when broths were plated onto 1/2 CCFA compared to CCFA. However these could in no way be confused with C. difficile being mostly small, pink colonies. Both the CCFA and 1/2 CCFA plates required 48h incubation before the C. difficile colonies were clearly identifiable.

3.2.1b Isolation of C. difficile from healthy adults

Three groups of volunteers were used in these studies (see Table 3.2). Faecal samples were used unfrozen and unrefrigerated (unless otherwise indicated) and all attempts were made to culture them less than 12h after collection.

Groups 1 and 2: About 2g of each sample was emulsified in 10ml of NB; 0.2ml of this was spread directly onto a CCFA plate as a control and 4.8ml inoculated directly into 100ml of CCFB.

Group 1: The remaining 5ml of homogenate was placed in a

TABLE 3.2

Groups of healthy volunteers providing faecal samples for study

Group	Males	No. on antibiotics	Ages	Females	No. on antibiotics	Ages
1	7	0	25-55	10	2	20-45
2	14	3	20-21	17	5	20-21
3	11	2	20-21	13	1	21

screw cap bottle and heated in a 70°C waterbath for 20 min; 0.2ml was spread onto CCFA and the remainder inoculated into 100ml CCFB.

Group 2: The 5ml of remaining homogenate was inoculated into 100ml of CCFB containing 0.1% sodium taurocholate (Sigma). For a later study 10 samples from this group which had been stored at -20°C were taken, 2g emulsified in 10ml NB and 5ml put directly into 100ml 1/2 CCFB. The remaining 5ml was added to an equal volume of ethanol and left for 1h before inoculation into a further 100ml of 1/2 CCFB.

These broths were incubated at 37°C and after 24, 48 and 72h a loopful from each was streaked onto a CCFA plate. This was also repeated after 7 days incubation of the broths. Samples inoculated into 1/2 CCFB were streaked onto 1/2 CCFA.

Group 3: Here the faecal samples were weighed and a loopful was spread onto 1/2 CCFA as a control. The entire sample was homogenised in 1/2 CCFB which was subsequently made up to 100ml with more 1/2 CCFB. Loopfuls from the broths were spread onto 1/2 CCFA plates after 24, 48 and 72h and assessed for growth of C. difficile after 24 and 48h incubation.

The first attempts to recover C. difficile from normal healthy adults were made with faecal samples (one sample per individual) from groups 1 and 2 cultured as described previously. The results obtained from these studies are summarised in Table 3.3.

C. difficile was not recovered from any specimen by direct inoculation onto CCFA. After enrichment the organism was cultured from three individuals none of whom were receiving, or had received antibiotics in the six months prior to sample collection. One of the males was 22, the other in his forties. The female was 23 years of age. The addition of the sodium taurocholate made no difference to the results. It took at least 48h enrichment in the CCFB to yield the organism. After seven days incubation, samples from the broths grew no C. difficile regardless of whether the organism had been isolated previously.

Heating was not found to increase recovery of the organism and in two of the three positive samples actually appeared to inhibit growth. The one heated culture yielding the organism also grew C. difficile from the untreated broth. Both these cultures required 72h incubation before the organism was isolated.

All broths inoculated with unheated samples grew many streptococci and showed signs of bacterial growth and fermentation within a few hours of inoculation. The broths inoculated with the heated samples did not show

TABLE 3.3

Recovery of C. difficile from healthy adults by culture in CCFB

	Number of C. difficile positive samples					
	Untreated broth		With heat		With Na^+ taurocholate	
Group	Male	Female	Male	Female	Male	Female
1	2	1	0	1	--	--
2	0	0	--	--	0	0

-- experiment not performed

such signs until 48 or 72h after the start of the experiment.

Ten samples found to be C. difficile-negative in this study were added to 1/2 CCFB with and without prior alcohol treatment. After 48h incubation of these broths the organism was isolated from another three samples. Two of these were from females (21 years of age and 34 years of age) who were receiving short term antibiotic therapy (tetracycline and amoxycillin respectively); the other from a female with no recent history of antimicrobial treatment (20 years of age). One of these strains was recovered only after alcohol treatment. The other two strains were from broths inoculated directly; the organism was not recovered after these two samples had been treated with alcohol. Plate contaminants were virtually eliminated after treatment with alcohol.

On the 1/2 CCFA plates several colonies grew that looked fairly similar to C. difficile. However after subculture to BA and CMB they were found to have different colonial morphologies and motility. Positive identification of C. difficile isolates was made by use of GLC and sugar fermentation tests.

3.2.1c The role of inoculum size in recovery of C. difficile from faeces

The work described previously was done with inocula of about 1g faeces into 100ml of enrichment broth. In order to assess further the role of inoculum size in recovery of the organism the following experiment was done. Two faecal specimens (1.5g each) from different healthy individuals, not receiving antimicrobials, were emulsified in 6ml of NB and 200µl of this suspension was inoculated into 30 x 10ml enrichment broths. Ten of these were CMBs, 10 were CCFBs and the remaining 10 were broths containing cycloserine, cefoxitin and mannitol (CCMB). All were incubated at 37°C. A loopful from each was spread onto CCFA after 24, 48 and 72h and the plates incubated for 72h before being discarded. One sample yielded no C. difficile in any of the broths; the other resulted in recovery of the organism from only four of the 30 broths. Three of these were from CCFB; the other from CCMB. All of the tubes grew quite high levels of streptococci.

From this result it appears that C. difficile must have been present at extremely low levels within this particular specimen. Clearly, there is a very real chance that the organism would not be recovered if any less than a gramme of faeces were used in trying to culture it from this sample.

Faecal samples obtained from individuals in group 3 (see pages 67 to 69) were weighed after collection and the entire sample (except a loopful that was spread directly onto 1/2 CCFA) inoculated into 1/2 CCFB. There were 24 samples weighing between 0.4g and 9.9g (see Table 3.4). C. difficile was not recovered from any of the samples when plated directly onto 1/2 CCFA. Only two of the 24 samples yielded C. difficile when the broths were cultured onto 1/2 CCFA after 48h incubation. One of these was from group ii, the other from group iv. Both the specimens were from 20-year-old males, one of whom had been receiving continuous antibiotic therapy (mostly tetracycline derivatives) for a year. The other had not been exposed to any antimicrobials. It would appear from these results that recovery of the organism is not increased by use of larger inocula sizes. Neither is there a gradation in levels of recovery with the different quantities of material cultured.

-

3.2.1d Summary of C. difficile isolations from healthy adults

As a result of all these studies C. difficile was recovered from eight of 72 (11%) individuals (one specimen per individual). Table 3.5 summarises how each of these isolates was obtained.

EDTA extracts were made from isolates 6 to 8 which had

TABLE 3.4

Samples used to investigate the role of inoculum size in recovery of C. difficile from faeces

Group	Weight of sample	No. of samples
i	less than 1.0g	3
ii	1.1g - 3.0g	8
iii	3.1g - 6.0g	7
iv	6.1g - 9.9g	6

TABLE 3.5

Method of isolation of C. difficile isolates from healthy individuals

	Method of culture				
Isolate	CCFB	1/2 CCFB	+heat	+alcohol	+Na taurocholate
1	+	...	+	...	-
2	+	...	-	...	-
3	+	...	-	...	-
4	...	+	...	-	...
5	...	+	...	-	...
6	...	-a	...	+	...
7	...	+	...	...	...
8	...	+	...	...	...

+: growth of C. difficile.

-: no growth of C. difficile.

a: isolate obtained only after alcohol treatment and culture in 1/2 CCFB.

...: method not tried for this isolate.

been isolated during the same investigation. All were shown to have different protein profiles when separated by SDS-PAGE (25µg). Isolate 1 and another isolate obtained from the same faecal sample during a subsequent study were examined in the same manner and found to be different, indicating this individual had been carrying two strains of the organism at the same time.

Cytotoxicity assays done on these eight isolates were all negative. All faecal samples from groups 1 and 2 were also tested for cytotoxic activity and all gave negative results.

Sera from seven individuals in Group 1 were tested for IgG antibodies against an EDTA extract of C. difficile NCTC 11223 by use of SDS-PAGE and immunoblotting. Extracts (25µg of protein in 50µl of double strength sample buffer) were immunoblotted with sera diluted 1 in 10 in antibody buffer (see section 2.18). Rabbit anti-human HRP conjugate (ICN Biomedicals - diluted 1 in 500) was used as the second antibody. None of the sera gave positive results despite isolation of C. difficile from two of these individuals.

3.2.1e Comparison of CCFA and 1/2 CCFA for routine direct isolation of C. difficile

It has been shown that the only disadvantage arising from use of 1/2 CCFA for isolation of C. difficile is the

greater number of contaminants that grow on it compared to on CCFA. However since other research groups have shown increased recovery of C. difficile with 1/2 CCFA the diagnostic laboratories here wanted to adopt this medium as the means for routine culture of the organism. An alcohol shock step was included in the isolation procedure to decrease contaminant growth.

One hundred unselected faecal specimens arriving in the diagnostic laboratories from general practice or hospital wards were examined. The samples were held at 4°C and cultured over the following few days. A loopful of each sample was inoculated directly onto both CCFA and 1/2 CCFA plates. The remaining specimen was homogenized in an equal volume of 50% ethanol (v/v) in NB with a vortex mixer and left on the bench for 60 min prior to inoculating a CCFA and a 1/2 CCFA plate with two loopfuls of this suspension. All four plates were subsequently incubated in the same anaerobic jar and examined after 48 to 72h.

Of these 100 specimens 16 yielded C. difficile. Five of the 16 organisms were obtained only on 1/2 CCFA. The rest were isolated on both. The use of alcohol did not increase recovery of the organism on CCFA but on 1/2 CCFA it increased isolation from 15 to 16 organisms. It did noticeably decrease the growth of plate contaminants.

A further comparison was done between the use of 1/2 CCFA with alcohol and the standard laboratory procedure for

isolation of C. difficile (direct plating onto CCFA). Consecutive specimens (265) were examined. Samples were held on the bench and cultured as a batch at the end of each day. Samples arriving during the weekend were held at 4°C until Monday. A loopful of each specimen was plated onto CCFA (standard isolation procedure). The remaining specimen was mixed with 50% ethanol (v/v) as previously described but for only 10 min. All other procedures were as in the previous experiment.

Of the 265 specimens 26 (10%) yielded C. difficile. All these isolates were cultured by the 1/2 CCFA/alcohol technique whereas only 19 grew on CCFA. A decrease in the period of alcohol shock from 1h to 10 min was found to be satisfactory for decreasing levels of contaminants.

In order to obtain a more quantitative idea of recovery on 1/2 CCFA compared to CCFA and also to determine the potential usefulness of alcohol treatment in the isolation procedure the following experiment was done. Two faecal samples (A and B) from which C. difficile had been previously isolated on CCFA were diluted 1 in 4 with NB. One half of each diluted sample was mixed with an equal volume of p-r NB and the remainder with an equal volume of absolute ethanol (final concentration therefore 50%). Samples were left at RT for 1h. Serial 10 fold dilutions were made of each sample and 50µl of each dilution spread in triplicate onto CCFA and 1/2 CCFA plates which were incubated for 48h before determining

the viable counts.

From the results (Table 3.6) it can be seen that there is a significant increase in isolation of C. difficile when the mean viable counts obtained by culture of alcohol treated and non-alcohol treated samples are compared (0.01 >P> 0.001). This would indicate that recovery of the organism from both faecal samples has been influenced by treatment with the alcohol.

The difference between the mean viable counts of organisms recovered by culture on CCFA and 1/2 CCFA does not appear to be so significant. For sample A (non-alcohol treated) and sample B (alcohol treated) there is no evidence to suggest any difference between the mean viable counts obtained (0.5 >P> 0.1). There is more likelihood of the 1/2 CCFA having increased recovery of C. difficile from the other two samples. However, the significance of the difference between these results is not as great as the effects observed during the comparison of alcohol treated and non-alcohol treated samples.

TABLE 3.6

Comparison of recovery of C. difficile on CCFA and 1/2 CCFA

with and without alcohol treatment

Sample	Mean Viable Count on[a] CCFA	1/2 CCFA	t test[d]	P value
A	2.9×10^6	2.7×10^6	0.93	0.5 >P> 0.17
A[b] (alc)	5.2×10^6	6.3×10^6	2.44	0.1 >P> 0.05
t test[c]	7.44	9.18		
P value	0.01 >P> 0.001	P> 0.001		
B	2.7×10^7	2.9×10^7	3.00	0.05 >P> 0.02
B[b] (alc)	3.4×10^7 ..	3.6×10^7	1.39	0.5 >P> 0.1
t test[c]	6.54	7.42		
P value	0.01 >P> 0.001	0.01 >P> 0.001		

see over/

a: mean of counts from three plates expressed as colony forming units per gramme of faeces. Appendix 3 records the counts from each individual plate.

b: figures obtained for alcohol treated samples.

c: t test comparing the significance of the difference between the mean values calculated for alcohol and non-alcohol treated samples.

d: t test comparing the significance of the difference between the mean values calculated for recovery of C. difficile on CCFA and 1/2 CCFA.

t test values were calculated with the following formula:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{SD^2}{n_1} + \frac{SD^2}{n_2}}}$$

where

$$SD^2 = \frac{\sum(x - \bar{x})^2_1 + \sum(x - \bar{x})^2_2}{(n - 1)_1 + (n - 1)_2}$$

SD = standard deviation
n = number of samples

P was calculated for the t values obtained by use of TABLE B in Statistics at Square One by T.D.V. Swinscow (1983).

3.3. Discussion

This work has confirmed that enrichment culture will increase the level of recovery of C. difficile from faecal specimens of normal, healthy individuals. During these studies the organism was not isolated from any individual when samples were plated directly onto CCFA or 1/2 CCFA but was recovered from 11% of samples after enrichment. Initial work indicated that about 10 vegetative C. difficile cells could be recovered from broths within 24h of inoculation while sporing cultures required 48h before recovery. With faecal samples all broths required at least 48h incubation before the organism was isolated. This was probably due to the organism being in the sporing form by the time it was cultured in the laboratory.

During these studies I used only samples that had not been refrigerated or frozen. George et al. (1982) have shown that freezing substantially reduces counts of C. difficile. Bowman and Riley (1986) found that C. difficile can remain viable in samples held at 5°C for up to 10 days. However, in an attempt to achieve maximum recovery I tried to use fresh samples that had been collected within 12h. I also asked all volunteers to provide as large a sample as possible to give any C. difficile present therein as much protection as possible from atmospheric oxygen. Bowman and Riley (1986) showed

that the larger a sample obtained, the better was the potential for survival of any C. difficile present.

There was no clear correlation between isolation of C. difficile and antibiotic therapy. Of the eight individuals from whom the organism was isolated (four males, 4 females) only three were receiving such agents (two on tetracyclines, one on amoxycillin). Although these antibiotics are associated with emergence of C. difficile (Bartlett, 1979) it should be borne in mind that a further 10 volunteers in these studies (all of whom were in their twenties) were also receiving such agents and the organism was not isolated from them. Also, any organisms isolated were present at low levels, all requiring enrichment before detection. Due to the fact that the volunteer population in these studies consisted mainly of individuals in their early twenties it is not possible to draw conclusions about any effect age might have on colonization with C. difficile.

Three isolates obtained during the same experiment were analysed by SDS-PAGE of their surface proteins and all were shown to be different. This would indicate that a variety of strains can exist within the normal healthy population and it also proved that the isolates had not arisen from a common contaminant within the anaerobic cabinet. It was also shown that one individual could harbour more than one strain of the organism at any one time.

All isolates obtained were non-cytotoxigenic. It would have been interesting to test for toxin A but at the time of this work pure anti-toxin was unavailable commercially for such an investigation. It is worth remembering that results obtained by toxin assay *in vitro* will greatly depend on cultural conditions employed during growth of the organisms. Haslam *et al*. (1986) clearly demonstrated that the precise environment of the gut at the time of isolation of the organism would require to be mimicked *in vitro* to get a true idea of the in vivo toxigenicity of an isolate. Clearly, such an ideal situation is impossible to obtain at present.

Of seven individuals tested for serum IgG antibodies against surface proteins of *C. difficile* all were negative, even two individuals from whom the organism had been isolated. It thus appears that these proteins are non-antigenic in these individuals when tested by this method. Use of an ELISA technique would perhaps have provided a more sensitive assay. I did not test for anti-toxins since a) no cytotoxic effect was detected and b) we did not have anti-toxin A.

What became increasingly apparent during this work was the potential role that inoculum size might be playing in recovery of the organism from samples. Enrichment studies in this laboratory had previously been done with 0.1g of faeces in 10ml of CMB. My original aims were to increase both the size of inoculum and the volume of culture

medium (to 1g in 100ml) and to provide a more selective broth to see if this would improve recovery of the organism from normal individuals. The study done in which 0.05g of a faecal sample was inoculated into each of 30 broths produced interesting results as the organism was recovered from only four of these. It had been thought that the composition of the broths might be important in determining the results obtained. As well as CCFBs and CMBs a broth containing mannitol (CCMB) was included. This could potentially have been more selective for C. difficile compared to CCFB as mannitol is fermented by fewer species than fructose. It was found however that only one of the four isolates was obtained from this CCMB; the other isolates were from the CCFB. From these results it seems that if only 0.1g of this sample were inoculated into a broth there would only be a 4/15 chance of recovering the organism (27%). It is clearly not feasible to try and culture C. difficile from any less than 1g of such a sample.

Considering these results I decided on a further study where much larger faecal inocula would be used. Based on the previous results it could be expected that larger inocula would result in increased recovery of the organism or even give a gradation in recovery with increasing inoculum size. However when this was done no such effect was encountered.

It was found that the addition of 0.1% sodium

taurocholate to broths or heat treatment of samples did not aid in recovery of the organism. In fact, heating samples appeared to inhibit growth of the organism. However, such results could be explained by the fact that the broths might not actually have received an inoculum of the organism if it were present at only low levels.

Again with the alcohol treatment there appeared to be some contrary results. When this was used to try and aid recovery of the organism from frozen samples it appeared that the treatment had again inhibited recovery of the organism. However, as previously, this may have been an inoculum effect. This is borne out by the fact that in both the routine isolation of the organism and culture of specimens known to contain high levels of C. difficile, alcohol treatment did noticably increase isolation of the organism. Such findings agree with other groups who have found the use of alcohol beneficial in recovery of C. difficile from faeces (Borriello and Honour, 1981; Levett, 1984b). Results from the quantitative study indicate that the alcohol treatment can increase the recovery of strains even when they are not inhibited by full strength CCFA. This could well be important if the organisms are present at quite low levels. The use of 1/2 CCFA will of course, be important in recovery of strains sensitive to higher antibiotic concentrations.

The original trials reported here, done in the diagnostic laboratories, used alcohol at a final concentration of

25%. Further studies showed that even one to two minutes treatment with 50% ethanol would give equally good results. Since then the following method, employing industrial methylated spirits instead of alcohol, has been fixed upon as being satisfactory for routine isolation of C. difficile from clinical specimens.

3.3.1. Recommended method for routine isolation of C. difficile from clinical specimens

1. Emulsify a pea-sized piece of faecal sample (about 1g) in 1ml of industrial methylated spirits and leave on the bench for one to two minutes.

2. Plate a loopful of this suspension onto 1/2 CCFA.

3. Incubate plates in an anaerobic cabinet where they can be easily checked for growth of C. difficile after 24h and/or 48h incubation. Plates incubated in an anaerobic jar are best left for 48h.

CHAPTER 4

EPIDEMIOLOGICAL STUDIES OF CLOSTRIDIUM DIFFICILE-ASSOCIATED DISEASE; CROSS INFECTION VERSUS ENDOGENOUS PATHOGEN ?

4.1. Introduction

The epidemiology of C. difficile-associated disease is an intriguing subject about which there are many questions but as yet very few clear answers. The organism appears pathogenic within the large bowel, almost always following some alteration of intestinal microecology. Although a few cases are reported with no apparent predisposing conditions or whose underlying disease is poorly understood, most patients have received antibacterial agents or some other treatment such as cancer chemotherapy. However, the risks associated with most of these therapeutic agents are not clearly defined.

4.1.1. Effects of antibacterial agents on normal gut flora

Agents capable of suppressing other gut anaerobes and of achieving high concentrations in the gut are the most likely to facilitate the acquistion and/or overgrowth of C. difficile (Larson et al., 1978; Wilson et al., 1981). Studies with antibacterial agent-treated animals confirm that the normal flora does play some protective role (Wilson et al., 1981). Rolfe et al. (1981) found that Lactobacillus, Streptococcus and Bifidobacterium species all suppressed growth of C. difficile in vitro. S. faecium was particularly inhibitory (Malamou-Ladas and Tabaqchali, 1982). Consequently it has been suggested

that removal of some of these normal bowel organisms by antibacterial agents could allow overgrowth of C. difficile. However it is clear that any ensuing disease cannot simply be due to drug-induced suppression of other gut flora since strains involved in disease are often susceptible in vitro to the agent being administered. In fact, isolates of C. difficile are always susceptible in vitro to ampicillin and frequently susceptible to clindamycin - even strains obtained from patients treated with these agents (Dzink and Bartlett, 1980). It is also unclear why some patients receiving antibacterial agents develop disease while others having the same treatment remain well. This may be partly due to differences in C. difficile strains as well as host or environmental factors (see Chapter 5). To date there is no evidence to suggest that the gut flora of patients who develop disease is any different or has an altered response to the predisposing agents administered compared to those individuals who remain healthy. The presumed role of the normal gut flora in protection is still poorly understood as are variations in the flora of normal persons and the specific effects of many therapeutic agents.

4.1.2. Potential reservoirs of C. difficile

There are two potential ecological niches for C. difficile. Either it exists somewhere within the environment or it is present as a commensal within human

beings and/or other animals. There are several reports of recovery of the organism from environmental sources (Mulligan et al., 1979; Malamou-Ladas et al., 1983); as already discussed the level of endogenous carriage of the organism within the human population is uncertain. It is of course, also conceivable that both endogenous and exogenous sources of the organism exist. If so, the relative importance of the two sources in the development of disease requires determination.

4.1.2a Environmental sources of C. difficile

Prolonged contamination of surfaces occurs readily in hospital environments (presumably due to the survival of highly resistant spores) especially if there is incontinence (Mulligan et al., 1980). Kim et al. (1980) recovered the organism in 15% of cultures from environmental sites 'potentially subject to faecal contamination' in the vicinity of three patients with C. difficile-associated diarrhoea. Only 3% of cultures in control sites were positive. Fekety et al. (1981) found the organism on hospital floors, buckets, toilets, bedding, mops, scales and furniture in the environment of patients with C. difficile-associated colitis. The organism was also present on these items, but less frequently, in areas where there were no known carriers. Carriage of C. difficile on the hands of hospital personnel has been documented (Mulligan et al., 1979; Kim

et al., 1981) and the organism has also been isolated from the faeces of asymptomatic staff (Fekety et al., 1981). Airborne transmission has not been conclusively demonstrated although Larson et al. (1980) suggested such spread of the organism might be possible when they found that animals housed in single cages, given clindamycin, developed disease symptoms before they had been handled or given food. Few studies have been done to investigate the possible distribution of the organism in environments other than those associated with hospitals and patients. The few reports which are available chiefly involve isolation of the organism from soil or peat (Martin et al., 1982).

4.1.2b Other potential reservoirs for C. difficile

Animals such as household pets have been reported as another potential reservoir for C. difficile (Borriello et al., 1982). Cytotoxic and non-cytotoxic strains of the organism were recovered from 21% of dogs, 30% of cats, as well as ducks and geese. The possibility of food-borne contamination has also been suggested (Gurian et al., 1982). However the organism has not as yet been detected in food products (Oishi et al., 1983) and it has been shown that at normal gastric pH levels C. difficile cells are rapidly killed and the cytotoxic activity is abolished. No mention was made of the proportions of vegetative cells to spores in this work (Gurian et al.,

1982). C. difficile has not been isolated from hospital foods or from unopened animal feed (Fekety et al., 1981; Kim et al., 1981).

4.1.3. Reported outbreaks of C. difficile-associated disease

C. difficile-associated PMC, AAC, AAD etc. could result from infection by another individual or from the environment. The most significant evidence for the infectious nature of these conditions is provided by the apparent clustering of cases in some instances which tend to suggest exogenous acquisition of C. difficile was an important step prior to development of symptoms.

Available evidence does indicate that PMC occurs as a sporadic infection. Swartzberg et al. (1977) saw 14 patients with PMC over a brief period then prospectively evaluated diarrhoea in 1000 patients given clindamycin and did not identify any more cases. The prospective study by Tedesco et al. (1974) identifying PMC in 20 of 200 (10%) consecutive patients receiving clindamycin is an experience confirmed by some (Kabins and Spira, 1975) but not by others (Ramirez-Ronda, 1974; Gurwith et al., 1977). Bennett et al. (1984) reported cross infection within a geriatric population where there was no isolation of the organism from the environment or from staff. The discovery of C. difficile as a specific aetiological factor in PMC and other bowel conditions has

permitted cases which occur under disparate circumstances to be associated. Consequently medical and surgical cases, antibiotic-associated outbreaks and clinically mild or severe cases can all be investigated and assessed together. When all the reports of C. difficile-associated disease are looked at what arises is a persistent flow of reports with grouped or clustered incidence. Greenfield et al. (1981) described eight cases in 11 days. This report has been followed by many similar accounts from centres worldwide, including London (Rogers et al., 1981; Haji et al., 1981), the USA (Pierce et al., 1982) and New Zealand (Ritchie et al., 1982).

What is lacking in all early epidemiological investigations is a reliable method for typing the isolated organisms to determine whether or not they are in fact the same strain. Clearly, if they were found to be different the case for cross infection would be substantially reduced. Burdon (1982) recovered a strain of the organism with distinctive antibiotic resistance from the majority of patients developing C. difficile-associated disease while in hospital. This resistance pattern was uncommon among isolates from other patients. Another early study (discussed later) involving typing of isolated organisms was done by Wust et al. (1982).

Both of these studies pointed to cross infection as the major method for acquisition of the organism. Now several

groups have been investigating a variety of methods for typing of C. difficile which will be discussed in greater detail in section 4.1.5.

4.1.4. Epidemiological studies of C. difficile in infants

One enigma when considering the epidemiology of C. difficile-associated disease is the fact that the organism is a common faecal isolate from healthy infants yet is rare in healthy adults. Is the latter a result of insensitive culture or is the former a consequence of exposure of an unusually susceptible population to a common environmental contaminant?

Some investigators have suggested that diet may influence the colonization of infants. One group (Cooperstock et al., 1982; Cooperstock et al., 1983) found that about 70% of formula-fed infants carried the organism compared to less than 20% of breast-fed individuals. Of these strains, about 50% were cytotoxigenic. Stark et al. (1982) reported earlier colonization of formula-fed infants and also that such infants carried higher levels of the organism. However, other reports have shown no such relationship (Holst et al., 1981; Brettle and Wallace, 1982; Richardson et al., 1983) or even that breast-fed infants have higher colonization rates (Donta and Myers, 1982).

It has also been suggested that the organism could be acquired from the mothers' vagina and/or cervix during

delivery (Gorbach et al., 1973; Hafiz et al., 1975). However, there is general scepticism about this as a method of acquisiton of C. difficile especially since many children acquire detectable numbers of the organism only days or months after birth. The organism is often isolated from infants while the mother is C. difficile culture-negative (Holst et al., 1981; Larson et al., 1982) and although vaginal delivery has been associated with increased faecal cytotoxicity in infants, the organism is also isolated from babies delivered by Caesarian section (Larson et al., 1982).

The available data on the levels of infant colonization by C. difficile shows considerable variability (Donta and Myers, 1982; Larson et al., 1982; Sherertz and Sarubbi, 1982). There is now increasing evidence indicating that the high levels of isolation reported by some groups may in fact arise as a consequence of person-to-person spread within hospitals. Given such a situation young children could subseguently acquire the organism as a result of their decreased resistance to infection.

Larson et al. (1982) undertook a prospective study in which 451 newborn infants were screened for the organism. This group thought that the previously reported high incidence of the organism in infants might in fact be the result of cross infection. Two studies at that time (Holst et al., 1981; Chang et al., 1979) had indicated that neonatal colonization could be less than 5%. Three

wards were studied by Larson et al. (1982) and colonization rates were found to range from 2 to 52% within these wards. Many colonizations were sporadic, but on two wards there was evidence of clustering. On one of these occasions prospective environmental sampling yielded C. difficile from a potential common source. Among the organisms tested there were cytotoxicity positive and negative isolates.

Sherertz and Sarubbi (1982) attributed the differing colonization rates reported in children to nosocomial spread of C. difficile and suggested that the organism could serve as a general marker for cross infection in neonatal intensive care units. It is worth noting that the variability observed in neonatal carriage of C. difficile is similar to the apparently sporadic development of hospital acquired AA-PMC, which again may point to a role for cross infection in the developement of disease.

4.1.5. Typing schemes for C. difficile

As mentioned previously most of the early work undertaken on the epidemiology of C. difficile-associated disease has relied on empirically obtained general impressions, lacking proper scientific evidence, as to the similarity or otherwise of the strains involved. Epidemiological studies would be greatly aided if a recognized system were available to 'type' any organisms isolated. Several groups have tried different approaches for developing a suitable, quick and reliable scheme and these are outlined below.

4.1.5a Antibiotic resistance patterns (antibiograms) and biochemical typing

A few groups (Rogers et al., 1981; Burdon, 1982) have attempted to trace the epidemiology of C. difficile-associated infections by determining the antibiotic sensitivities of the strains isolated. However, such studies are of limited usefulness since antibiotic resistance within an organism tends to be widespread. Bolton et al. (1984) used antibiogram typing during a study of neonatal colonization but admitted it was of limited usefulness.

Poxton (1982) arranged 43 C. difficile isolates into seven different biotypes based on their reactions in six biochemical tests. Some of these results took a long time to obtain and some sugars were fermented only weakly.

Since this work other techniques have been developed which provide much quicker and clearer definition between strains.

4.1.5b Use of bacteriophage and bacteriocins

Sell et al. (1983) were able to isolate 10 bacteriophages and 20 bacteriocins active on C. difficile. Of 254 organisms studied, 132 were found to be susceptible to phage or bacteriocin. Within the phage sensitive isolates at least nine distinct groups could be discerned. With these phages and bacteriocins various isolates cultured from patients at different times were investigated and found to be similar while isolates from different geographic locations were found to vary. It would seem that this technique has potential as a reliable method for typing strains.

Wust et al. (1982) compared 16 isolates obtained during a hospital outbreak of C.difficile-associated colitis by CIE, TCA, and analysis of plasmid and SDS-PAGE profiles. Of these isolates 13 resembled each other so closely when compared they were classed as the same strain although there were minor differences between some of them. It was concluded that this strain had been spread by cross infection during the outbreak. Hachler and Wust (1984) analysed these same 16 organisms by use of bacteriophage typing. Ten of the 16 were found to be susceptible to one particular phage but not to any of the others available.

The other three isolates originally thought to be the same strain, were found to be resistant to all of the available phages and it was concluded that they were unlikely to have been involved in the outbreak. The other three strains were also shown to be resistant to all the phages.

4.1.5c Serum agglutination

Nakamura et al. (1981b) showed that formalin treated strains of C. difficile could be agglutinated with rabbit antisera. Delmee et al. (1985) took this approach further and produced six different agglutinating antisera (in rabbits) which they used to define six serogroups of C. difficile. An excellent correlation was found between the serogroup and sorbitol fermentation by strains. It was found that certain of these serogroups were isolated from outbreak cases of AAD involving both adults and children (A, C and D). Different serotypes were shown to be carried by asymptomatic neonates (B, F and G). Other epidemiological studies have now been done with this method (Delmee et al., 1986; Delmee and Michaux, 1986; Mulligan et al., 1986). Serogrouping has the potential for development into a very quick and simple typing method which would be useful in the diagnostic situation. However, for such a system to be fully comprehensive, a wide variety of strains need to be investigated and an adequate array of antisera produced.

4.1.5d Use of electrophoresis

i) CIE: Poxton and Byrne (1981b) showed that four different precipitin lines could be visualised when EDTA extracted surface antigens of C. difficile were separated by CIE. It was shown that these lines occurred with differing intensities in different isolates and it was thought use of CIE in conjunction with biotyping might prove useful for studying C. difficile outbreaks. However this has now been abandoned in favour of other techniques giving more objective results.

ii) SDS-PAGE: the earlier investigations using this technique relied upon simple comparison of complex protein profiles produced by whole cell extracts of isolates (Cato et al., 1982; Kim et al., 1983). Wexler et al. (1984) examined EDTA extracts of isolates, placing emphasis on the presence or absence of particular protein bands within an extract. This approach was also adopted by Mulligan et al. (1986).

Tabaqchali et al. (1984a) used sulphur-35-labelled methionine to radiolabel isolates and help differentiate between strains. This procedure gives rise to patterns of radiolabelled proteins which can be visualised by autoradiography. Based on analysis of such gels nine distinct profiles (labelled A, B, C, D, E, W, X, Y and Z) were obtained among isolates from an outbreak of C.

difficile-associated disease. Like Delmee et al. (1985) certain strains were isolated from asymptomatic neonates (A to D) while strains isolated from clinical outbreaks of AAC among adults belonged to groups X and E. Only five strains out of 250 studied were non-groupable. This group have continued to use this technique to demonstrate cross infection and nosocomial acquistion of the organism (Heard et al., 1986a).

Poxton et al. (1984) examined EDTA extracts of C. difficile isolates separated by SDS-PAGE. Coomassie blue stained gels were looked at in conjunction with immunochemical analysis by electroblot transfer (immunoblotting). In this way it was possible to identify one strain of C. difficile involved in a hospital outbreak of C. difficile-associated disease in Sweden and to differentiate this from several other isolates.

Mulligan et al. (1986) did a comparison of results obtained by SDS-PAGE of EDTA extracts and bacterial agglutination of cells to distinguish isolates of C. difficile. Excellent correlation was found between the two methods when geographically distinct isolates were typed without knowledge of their clinical origin.

4.1.5e Whole cell DNA restriction endonuclease profiles

Kuijper et al. (1987) obtained unsheared chromosomal DNA from C. difficile, free from plasmid DNA, which was subsequently digested with Hind III. Repeat preparations

from the same isolates gave reproducible results. The technique has been used to show that six isolates of C. difficile (two from patients with PMC; four from environmental sampling) were identical.

4.1.6. Conclusions from studies done with typing techniques

It would seem that the introduction of various typing schemes has further emphasized the possibility that C. difficile can be spread from one individual to another in an epidemic fashion (Heard et al., 1986a; Hawkins et al., 1984). However, various teams have also found that the typing of isolates indicates that cross infection (in their particular studies) has not been important (Rampling et al., 1985; Hall et al., 1985). It should be remembered that any epidemiological investigation requires confirmation that increases in recovery of the organism are truly the result of an outbreak and not spurious causes such as better identification schemes or sudden laboratory interest in the organism (Hall et al., 1985). For this reason prospective studies are particularly useful.

Unfortunately, few of these have been reported to date. Heard et al. (1986a) have published results of one prospective study involving a large-scale clinical outbreak of C. difficile-associated diarrhoea. Following ^{35}S-methionine labelling of cells and subsequent

autoradiography it was shown that this outbreak was most likely due to cross infection by one particular strain of C. difficile (type X). This was cultured from 35 of 49 (71%) oncology patients from whom C. difficile was isolated. In addition 29 patients who were C. difficile-negative on entry into hospital acquired this strain during their stay.

In conclusion, it can be said that there is still much confusion over the relative roles of cross infection and/or endogenous infection by C. difficile. Perhaps now that laboratories are, in general, more aware of the organism and typing schemes are becoming available more information will become available on which to try and base conclusions.

4.1.7. Aims of this study:

1.a) to assess the antigenic stability of C. difficile during different growth conditions and EDTA extraction of surface proteins. As a consequence of these studies it would be possible to assess the reliability of immunoblotting for use in differentiating isolates.

1.b) to produce a set of guidelines for the successful use of immunoblotting in epidemiological investigations.

2. to study any 'outbreaks' of C. difficile-associated disease to assess the importance of cross infection.

It was also hoped to determine whether an individual could harbour more than one strain of the organism at any one time.

4.2. Results

The following studies were done to investigate the antigenic stability of C. difficile, both during culture and subsequent antigen extraction of isolates. The organisms used in these investigations were isolated from renal patients involved in an epidemiological investigation occurring at that time (see section 4.2.5a) or from other medical patients in the Royal Infirmary of Edinburgh (RIE). In all studies 25µg of protein in 50µl of double strength sample buffer was applied to each track of the SDS-PAGE gels. Immunoblots were all probed with antisera raised against whole cells of NCTC 11223.

4.2.1. Effect of variation in phase of growth at harvesting on the immunoblot patterns produced

With the standard technique organisms are harvested and antigens extracted with EDTA buffer after 16 to 18h cultivation (see section 2.19). For this study a 5% inoculum of MPRL 558 was cultured in PPY and 100ml amounts were harvested after 2, 4, and 8h. Similarly a 1% inoculum was harvested after 16, 18, 20, 24 and 40h. All other conditions remained the same as in the standard technique during subsequent EDTA extraction.

These extracts were all compared by SDS-PAGE and immunoblotting. From the subsequent immunoblot patterns produced (Fig. 4.1a) it can be seen that there was a

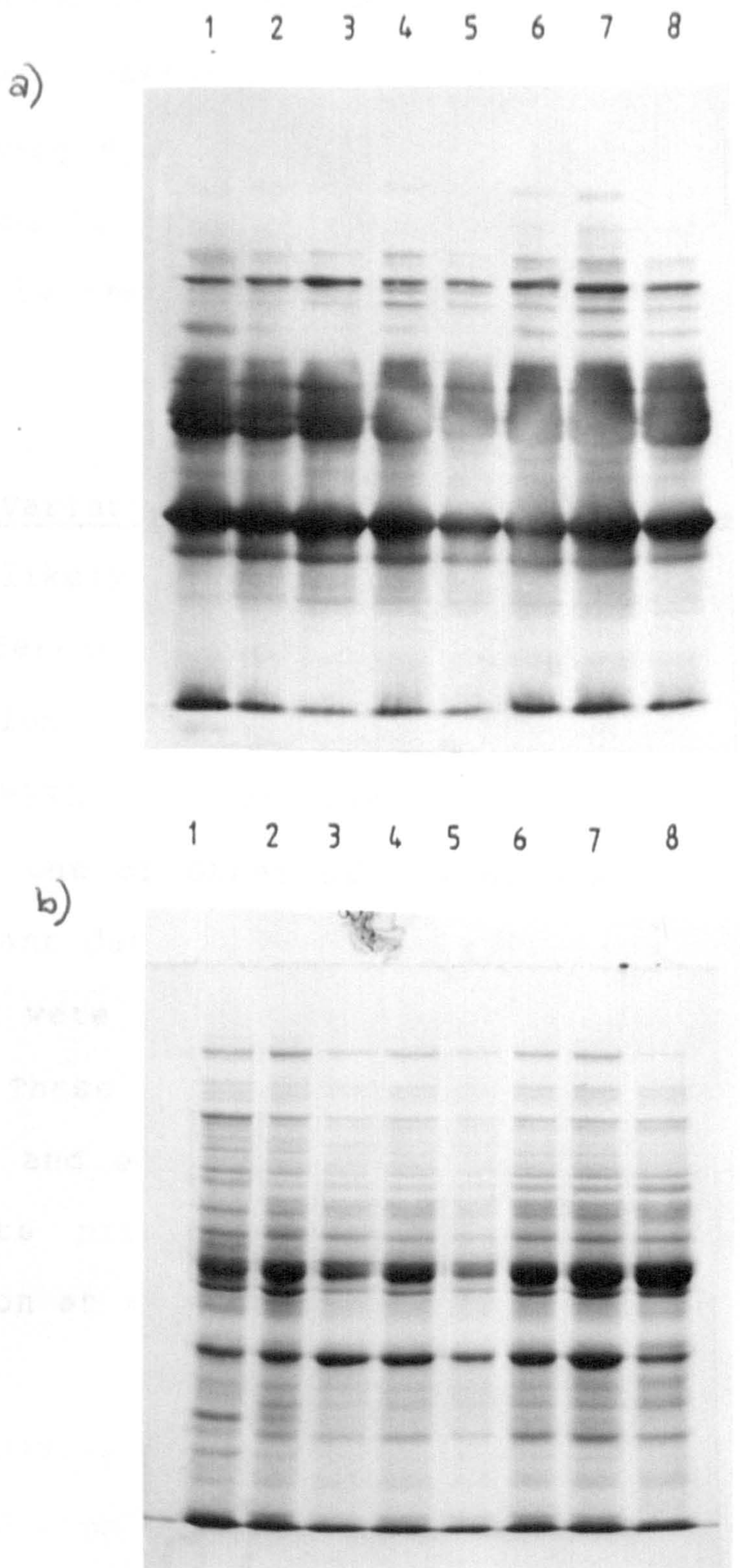


Fig. 4.1. (a) Immunoblot patterns produced by EDTA extracts of C. difficile MPRL 558 prepared 2 to 40h after inoculation of PPY medium. Tracks 1 to 8 (25µg of protein per track) contain extracts obtained after 2, 4, 8, 16, 18, 20, 24 and 40h respectively. Extracts were probed with antiserum raised to NCTC 11223 diluted 1 in 250 with antibody buffer.
(b) Corresponding SDS-PAGE profile stained for protein with Coomassie blue.

great degree of similarity in the banding patterns. There were minor variations, especially in the polypeptides with higher M_rs, but these may in part be due to slight variations in the amount of protein loaded onto the gel as seen in the corresponding Coomassie blue stain (Fig. 4.1b).

4.2.2. Variations in antigen extraction

Another likely source of variation between extracts could be differences in the actual method of antigen preparation. Three C. difficile isolates (MPRL 558, MPRL 559 and MPRL 589) were used to investigate the effects of altering one or other of the parameters usually held to be constant during preparation of EDTA extracts.

Cultures were harvested, washed and resuspended in EDTA buffer. These suspensions were then divided into four aliquots and each was subjected to one of the following treatments prior to harvesting: (a) 'normal' antigen extraction at 45°C for 30 min, (b) extraction at 50°C for 30 min, (c) extraction at 45°C for 2.5h, (d) freeze-thawing (-20°C to 37°C) three times over a period of 3h and then 45°C for 30 min.

When these extracts were analysed it was found that MPRL 558 and MPRL 559 produced very similar banding patterns when stained with Coomassie blue. However there were consistent differences between the isolates in the immunoblot patterns produced (Figs 4.2a and 4.2b). There

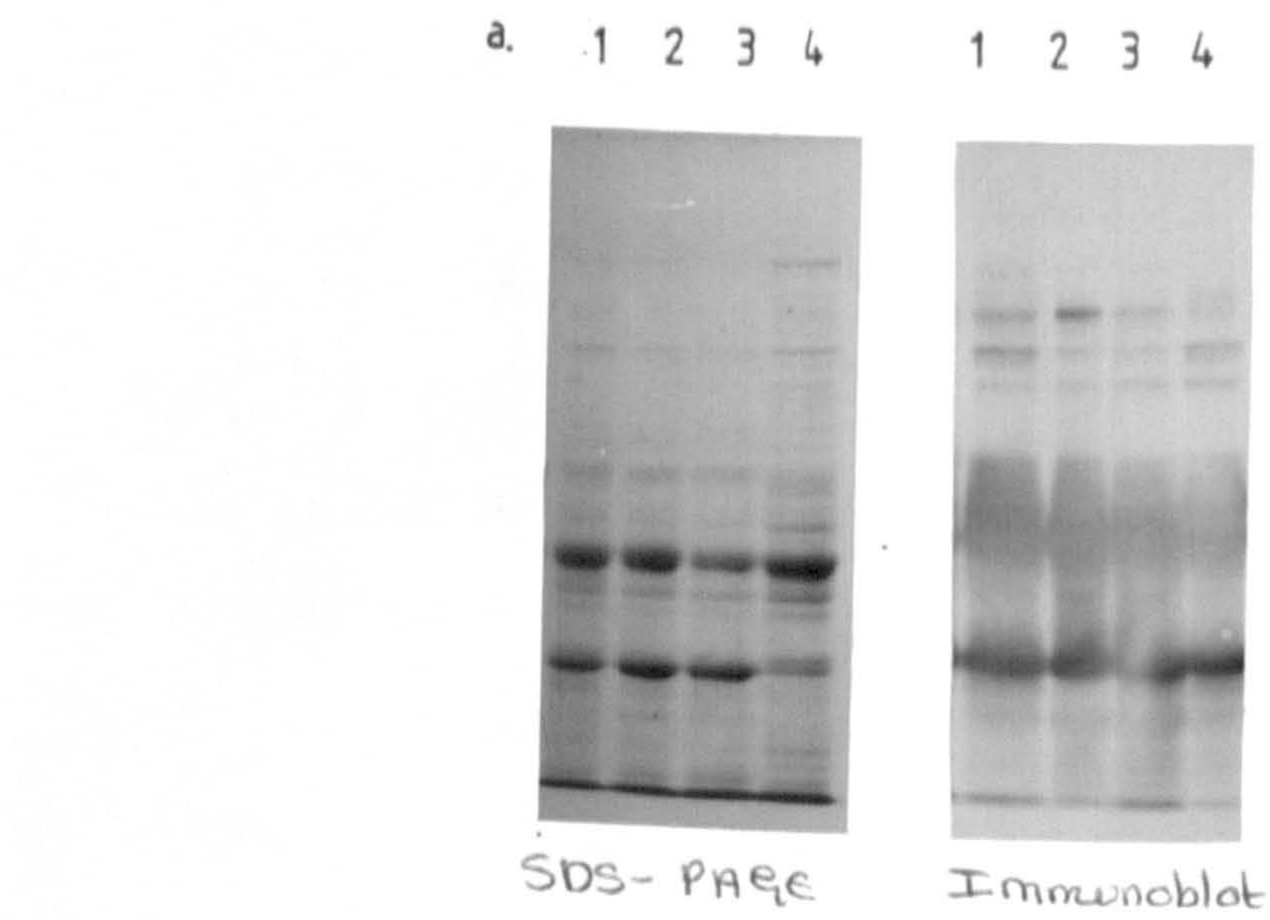


Fig. 4.2. SDS-PAGE and immunoblot patterns produced by (a) MPRL 558, (b) MPRL 559 and (c) MPRL 589 after various treatments during EDTA extraction. Track 1: extraction at 45°C for 30 min, track 2: 50°C for 30 min, track 3: 45°C for 2.5h, track 4: freeze-thawed extract.

was a much stronger reaction involving the high M_r proteins in MPRL 559 than occurred with MPRL 558 and this remained consistent despite the differing treatments during extraction. MPRL 589 behaved differently. Here the extract which had been heated for 2.5h produced a blot pattern which was different from the others in the mid-region of the track (Fig. 4.2c). Why this occurred is not clear. With all three isolates the EDTA/freeze-thawed extracts had more protein bands apparent on the Coomassie stained gel. However, the immunoblot did not show up these additional bands indicating that they were probably intracellular proteins with which the antisera raised against whole u-v killed bacteria would not react.

4.2.3. Effect of differing periods of development on the immunoblot patterns produced

Four immunoblots of EDTA extracts of MPRL 832, MPRL 835 and MPRL 840 were prepared and placed into developing solution. One was removed after 1.5 min, another after 3.5 min, one after 10 min and the final one after 16 min. It was found that the longer the immunoblot was left to develop, the stronger the colour of the bands became (Fig. 4.3). Some bands, not apparent after 1.5 min, appeared after longer periods of development. Also, the amount of background staining increased the longer the blot was left. A Coomassie stain of these same isolates suggested that tracks 2 and 3 were identical but the

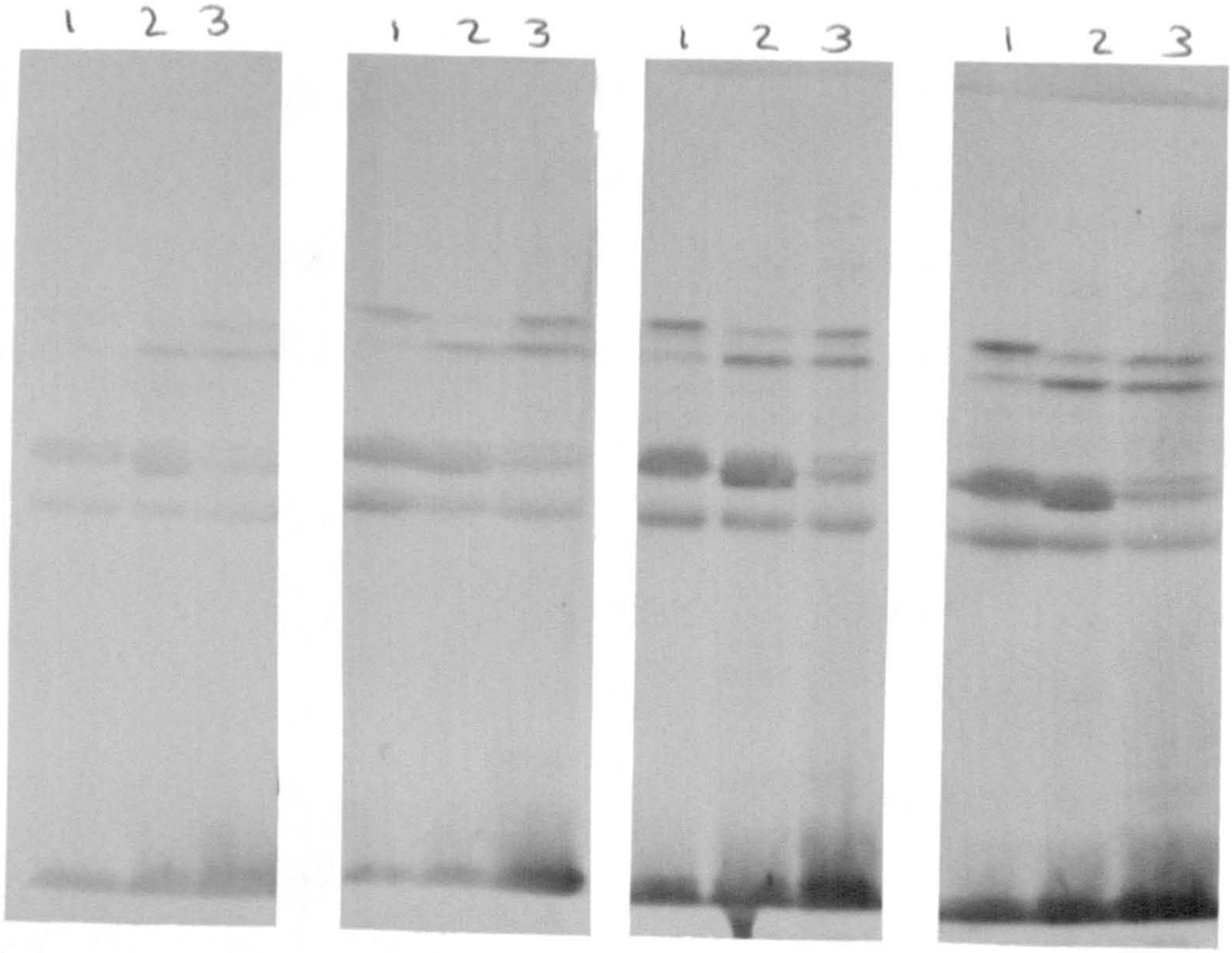


Fig. 4.3. Immunoblot patterns resulting from differing periods of time in HRP colour development solution. (a) was left for 1.5 min, (b) for 3.5 min, (c) for 10 min and (d) for 16 min before stopping the reaction. Tracks 1 to 3 contain EDTA extracts of MPRL 832, MPRL 825 and MPRL 840 respectively.

immunoblot shows up subtle differences between the two isolates. This is not really apparent until after 10 to 16 min development of the blot.

4.2.4. Variations on subculture of the organisms

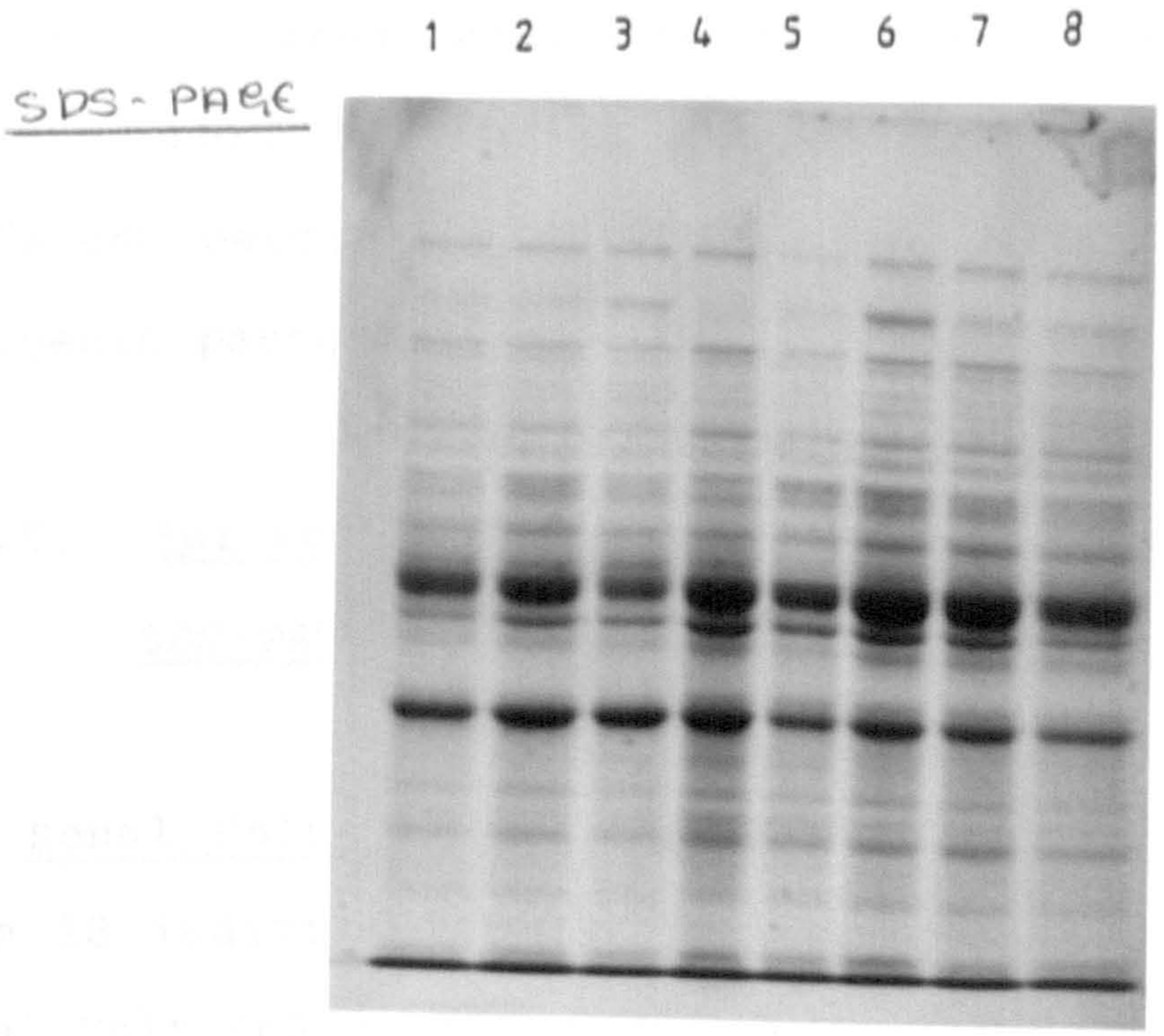
Having found no major variations in immunoblot pattern could be accounted for by differences in culture or antigen preparation, further studies to determine the stability of an individual isolate both in vitro and in vivo were undertaken.

in vitro: a CMB culture of MPRL 558 was passaged weekly into fresh p-r CMB over seven weeks. EDTA extracts were prepared from each of these by the standard method after incubation for 24h.

in vivo: a spore suspension of MPRL 558 (100µl of saline containing 10 C. difficile colonies from a 72h BA plate) was administered orally to a female Balb/c mouse known to be C. difficile-negative after faecal enrichment in CCFB. Faecal pellets were collected weekly for seven weeks. Each was inoculated into 10ml p-r CCFB which was incubated for up to 72h. EDTA extracts were prepared of the organisms isolated on CCFA.

When the resulting sets of extracts were analysed the patterns produced were shown to be identical over the entire period of study(e.g. Fig. 4.4 - patterns from in vivo isolates).

The only other potential source of variation during this work was in the composition of the PPY medium used to



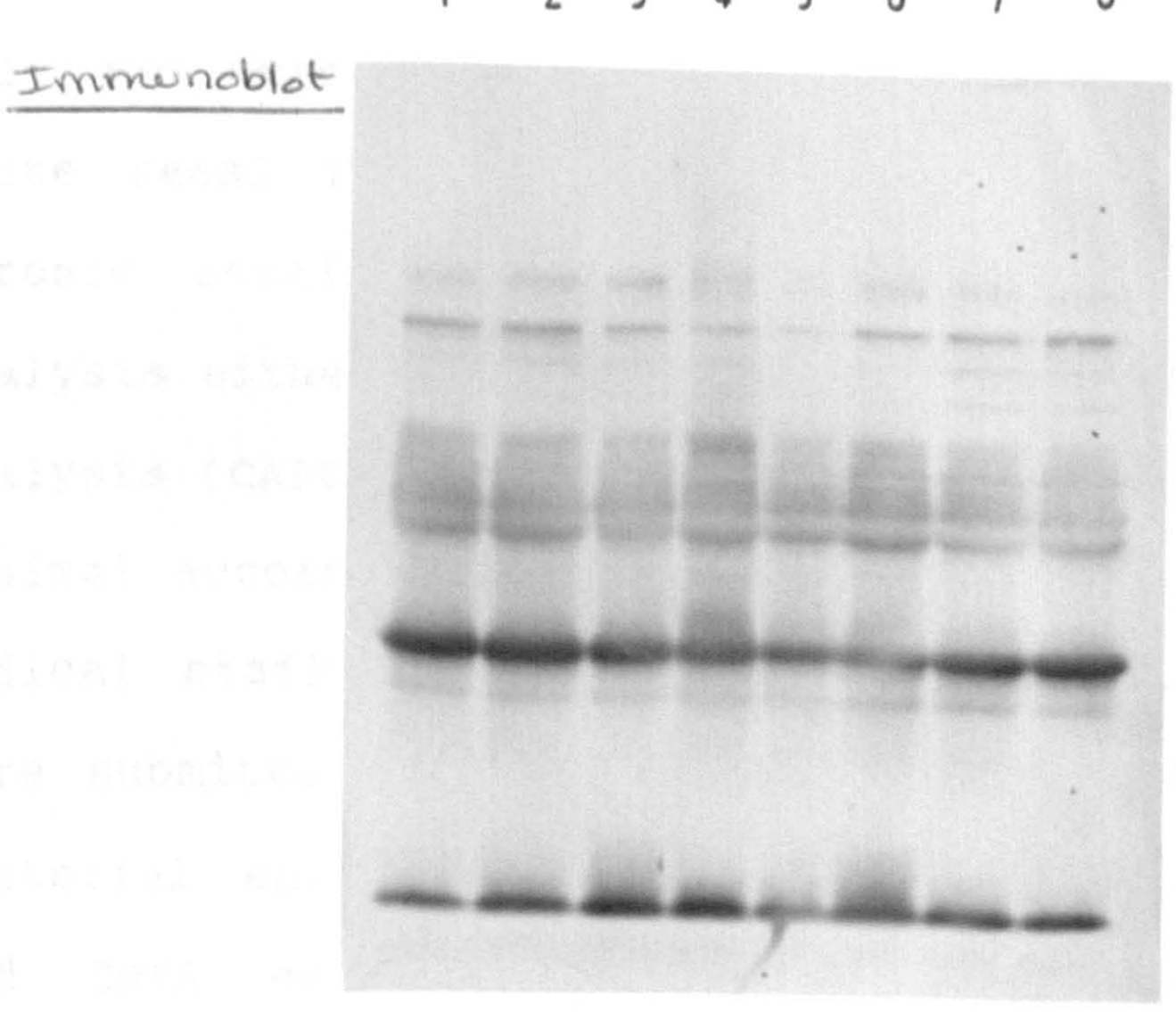


Fig. 4.4. SDS-PAGE and immunoblot patterns produced by EDTA extracts of MPRL 558 obtained from faecal pellets of a Balb/c mouse. Tracks 1 and 2 contain extracts of isolates cultured after 24h and 4 days. Tracks 3 to 8 are from weeks 2 to 7 respectively.

culture the organisms. However, these studies were done over a period of four months during which several different batches of media were used and no variation in antigenic patterns was observed.

4.2.5. Epidemiological investigations undertaken using SDS-PAGE and immunoblotting

a) Renal Unit Investigation: C. difficile was isolated from 18 individuals who were in-patients in the Medical Renal Unit (MRU) at the RIE between August 1983 and April 1984. Details of these individuals are given in Table 4.1. Two were undergoing haemodialysis (HD) because of acute renal failure. The other patients had end-stage chronic renal failure and were receiving maintenance dialysis either by HD or continuous ambulatory peritoneal dialysis (CAPD). Diarrhoea was graded severe, moderate or minimal according to patient interview or observation by medical staff. Stool specimens from all these patients were submitted to the laboratory for routine culture of bacterial enteric pathogens. C. difficile was isolated and EDTA extracts of surface proteins prepared as described in section 2.19.

Fig. 4.5 shows the results of Coomassie blue staining and immunoblotting of SDS-PAGE gels. It can be seen that there are a variety of protein profiles produced on staining and that some isolates appear very similar. Use

TABLE 4.1 Details of patients from whom C. difficile was cultured

Patient	Isolate (MPRL)	Age (yrs)	Sex	Type of dialysis	Severity of diarrhoea	Type of infection	Antibiotics administered	Month of isolation		Outcome
1	559	61	F	CAPD	+	Peritonitis	None	July	1983	Resolved
2	558	60	F	CAPD	+++	Peritonitis	Crd,Flu,Tob	"	"	Died
3	560	48	F	HD	-	None	None	"	"	Remained Well
4	589	15	F	CAPD	++	Peritonitis	Crd,Tob	Aug	"	Died
5	595	56	F	HD	+++	Wound	Cfx,Met	"	"	Diarrhoea Continued
6	597	59	F	HD	++	AV-fistula	Flu,Pen	"	"	Resolved
7	604	61	M	CAPD	+	Peritonitis	Flu,Met,Tic	"	"	Resolved
8	616	69	F	CAPD	+	Peritonitis	Crd	Sep	"	Resolved
9	629	59	M	HD	+	Mastoid	Flu,Pen	Oct	"	Resolved
10	681	50	M	HD	+	Pericolic abscess	Crd,Cfx,Met	"	"	Resolved
11	678	68	F	CAPD	++	Peritonitis	Tob	Nov	"	Resolved
12	659	71	F	CRF	+	None	None	Jan	1984	Resolved
13	665	73	M	HD-acute	+++	Pneumonia	Amp,Cfx,Ery, Met,Gen,Pen, Cot	"	"	Resolved
14	816	33	F	HD	++	Urinary tract	Cot	Feb	"	Resolved
15	694	63	F	HD-acute	+++	Ischaemic bowel	Cfx,Met,Tob	"	"	Died
16	696	64	F	CAPD	++	Peritonitis	Flu	"	"	Resolved
17	712	60	M	CAPD	+	Peritonitis	Flu	Mar	"	Resolved
18	718	66	F	HD	+++	AV fistula	Crd,Cfx,Tob	"	"	Died

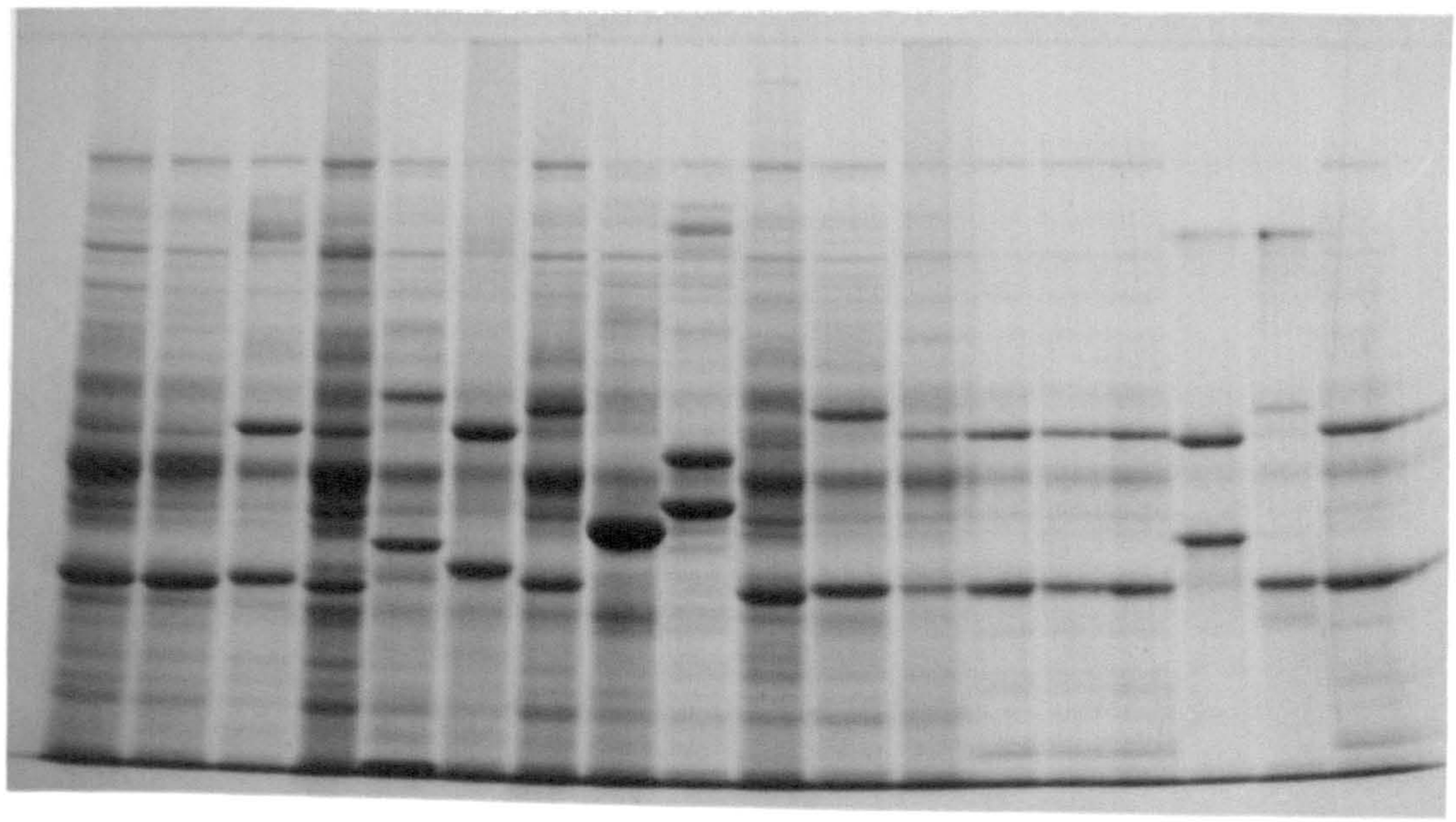
Type of dialysis:
CAPD: continuous ambulatory peritoneal dialysis
HD:haemodialysis
CRF: chronic renal failure not yet on dialysis

Symptoms: +++ severe diarrhoea; ++ moderate diarrhoea; + minimal diarrhoea; - no diarrhoea

Antibiotics administered:

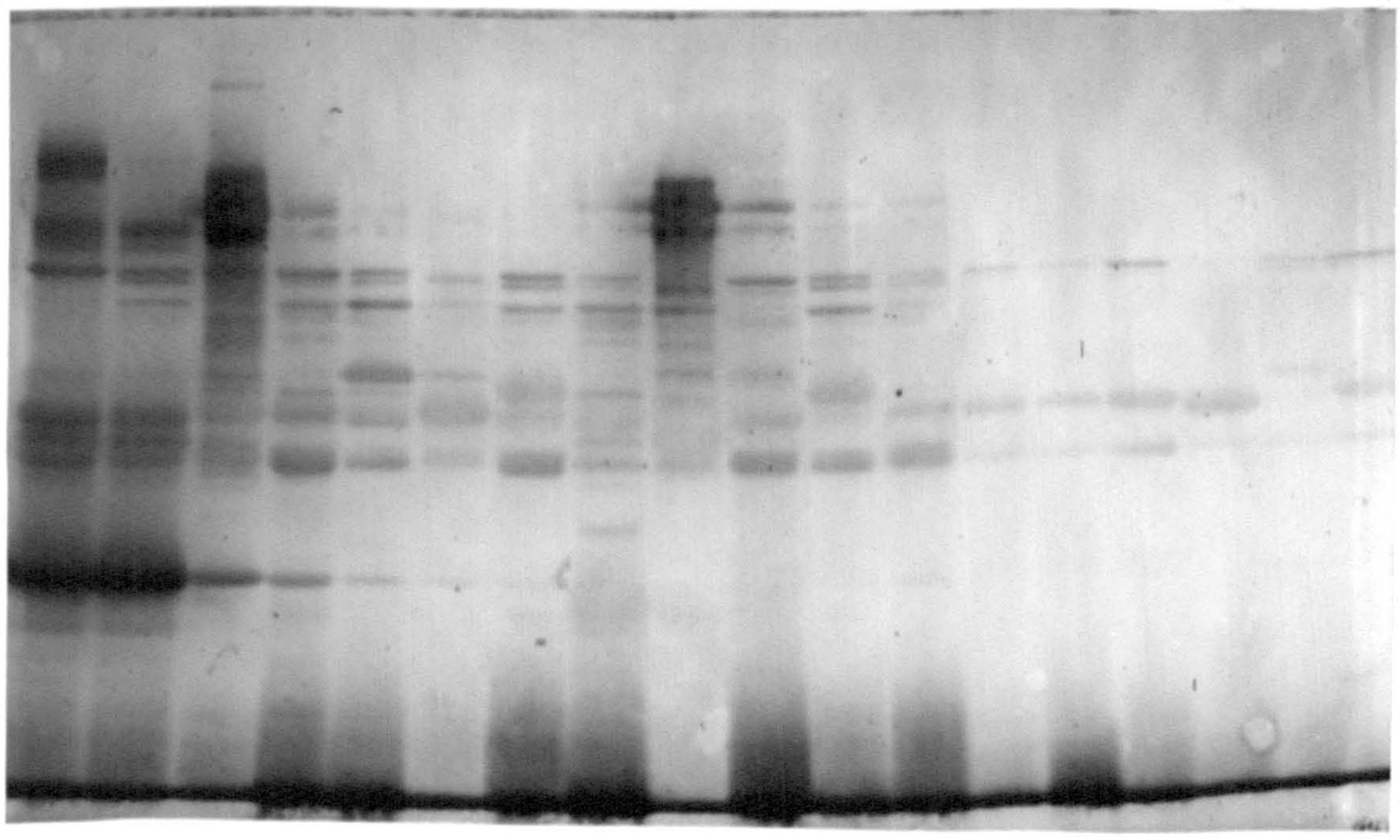
Amp: ampicillin	Gen: gentamicin
Cfx: cefuroxime	Met: metronidazole
Cot: co-trimoxazole	Pen: benzylpenicillin
Crd: cephradine	Tic: ticarcillin
Ery: erythromycin	Tob: tobramycin
Flu: flucloxacillin	Van: vancomycin

a.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

b.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 4.5. SDS-PAGE of EDTA extracts from the 18 strains of C. difficile isolated from the MRU patients listed in Table 4.1. (a) stained with Coomassie blue and (b) corresponding immunoblot probed with antiserum to NCTC 11223.

of the immunoblotting technique (Fig. 4.5b) confirmed that at least 13 distinct profiles were produced. The same pattern was produced by isolates from five individuals (Nos 12, 13, 14, 15 and 18). The first four of these patients had been in the MRU at the same time; patients 15 and 18 subsequently died although they were both severely debilitated because of pre-existing medical conditions (ischaemic bowel and polyarteritis nodosa respectively).

Another strain was isolated from two patients (Nos 7 and 11); the strains isolated from patients 1 and 2 were also very similar on the Coomassie stain but there were consistent differences in the immunoblot patterns produced especially with the high M_r proteins.

Having established that a variety of strains was involved in this outbreak of C. difficile-associated disease in these patients a further study was set up to find whether two or more different strains might co-exist in the gut at any one time. Two faecal samples were used (X and Y), both from MRU patients who had not been included in the original study. C. difficile (MPRL 720 and MPRL 808) had been isolated from these specimens previously. The patients had also had the organism isolated from them on two other occasions.

Ten separate colonies were picked from a primary CCFA isolation plate and EDTA extracts prepared from each of these. Eight of the extracts produced from C. difficile

colonies isolated from sample X gave two distinct profiles (Fig. 4.6). One of these profiles (in tracks 1, 2, 3, 6 and 7) corresponded to that produced in a previous gel by MPRL 720. All 10 extracts from sample Y were run and one of those was shown to be different from the others. Both patterns produced were different from two other strains also isolated from this patient on other occasions.

4.2.5b Investigation of Dutch Isolates: Six isolates of C. difficile were sent to the laboratory from Amsterdam where they had been isolated from a suspected incident of cross infection between two patients (Table 4.2). Both of these patients had received metronidazole, tobramycin and ampicillin although we were informed that the isolated organisms were sensitive to the last of these. Patient A had PMC (as diagnosed by colonoscopy); patient B had fever, abdominal pain and watery diarrhoea but no sign of pseudomembranes. When examined by immunoblotting it was apparent that all these isolates were the same. It is therefore entirely possible that they could have been transmitted between the patients. It was noted at the time of isolation of these organisms that there was no increase in rate of isolation of C. difficile elsewhere in the hospital.

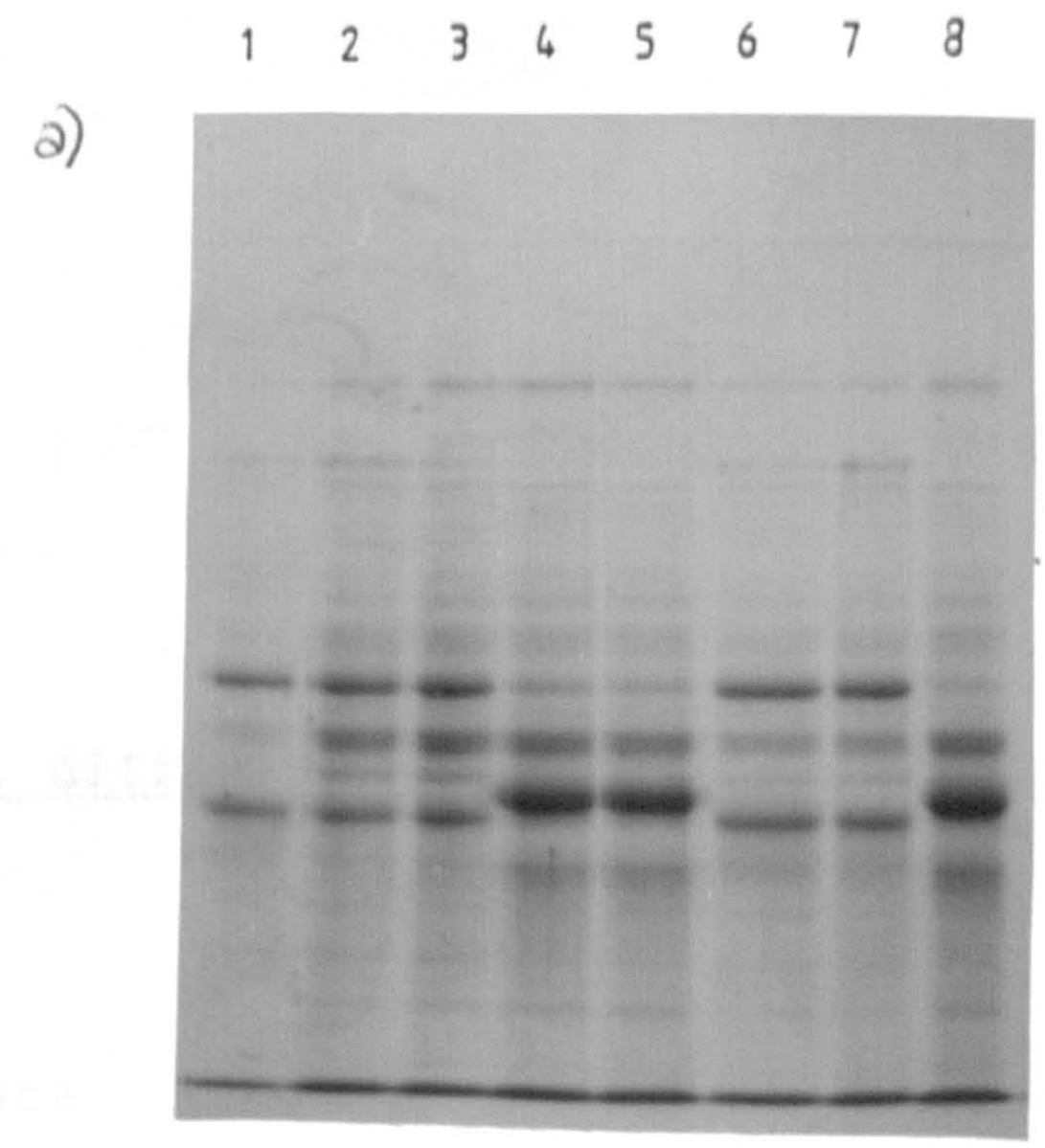


Fig. 4.6. Analysis of EDTA extracts prepared from 8 different colonies picked from a CCFA primary isolation plate. (a) SDS-PAGE, (b) immunoblot.

TABLE 4.2

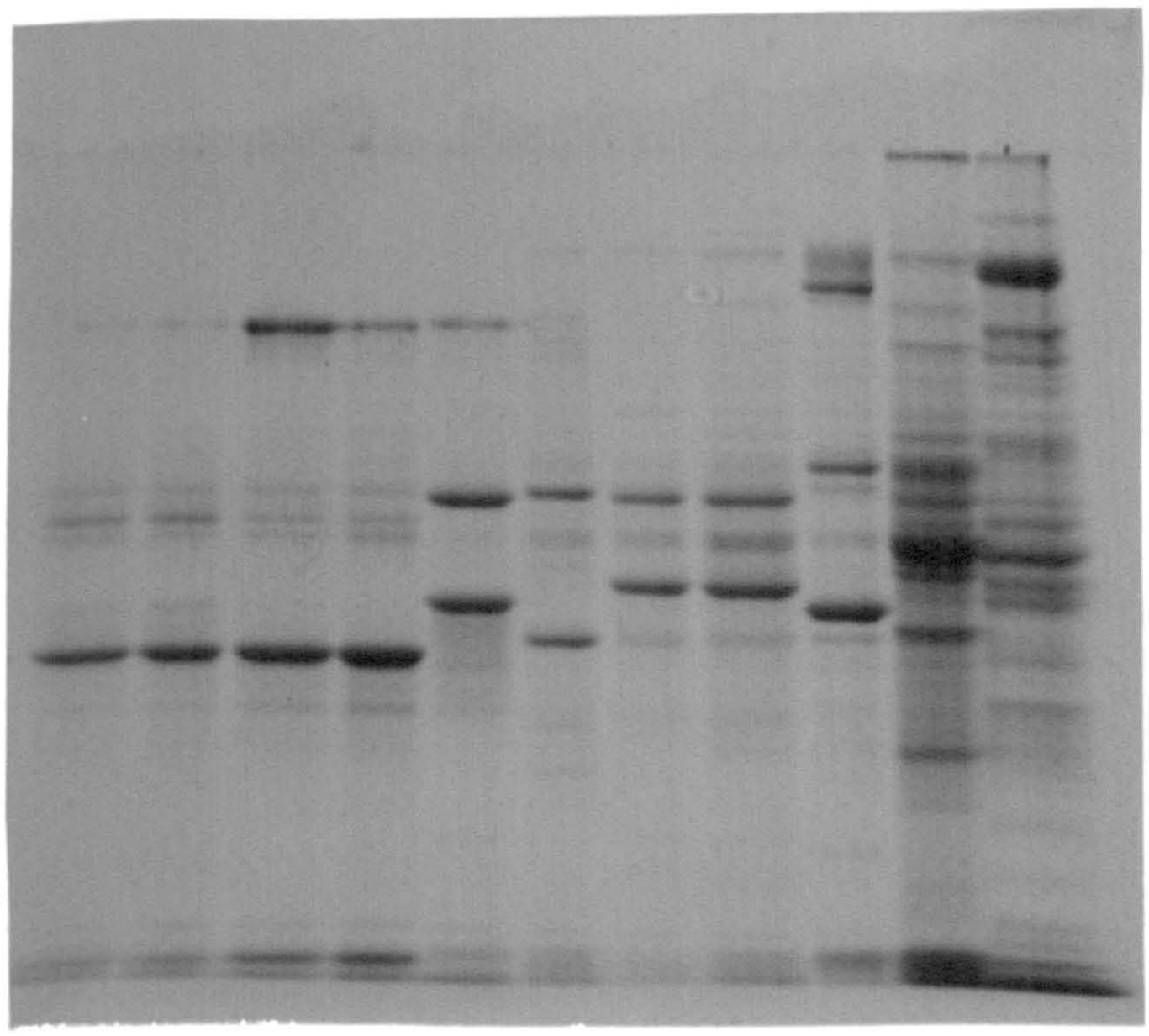
C. difficile isolates from Amsterdam

Isolate	Source	Date of Isolation
MPRL 1169	Patient A	2nd April 1985
MPRL 1170	Patient B	4th " "
MPRL 1171	floor	8th " "
MPRL 1172	telephone	8th " "
MPRL 1173	floor	17th " "
MPRL 1174	floor/corridor	24th " "

4.2.5c Investigation of Irish isolates: We were sent 11 organisms which had been isolated from patients suffering from PMC in three Dublin hospitals (see section 2.1. - list of bacterial strains). The isolates were obtained over a period of 12 to 13 months. Cross infection between patients was a possibility since there were frequent transfers between the hospitals.

When analysed four of these isolates were found to be identical (tracks 1 to 4, Fig. 4.7, containing MPRL 1244, MPRL 1245, MPRL 1246 and MPRL 1247). The two strains in tracks 7 and 8 (MPRL 1250 and MPRL 1251) were also shown to be the same. The isolate in track 11 looked very different from the others on the Coomassie stained gel and there was no reaction apparent on the immunoblot. The extremely high contrast film used for the photography actually gives a false impression of the reaction. When its biochemical and fermentation patterns were checked it was found that this isolate was in fact a Clostridium butyricum.

a.



b.

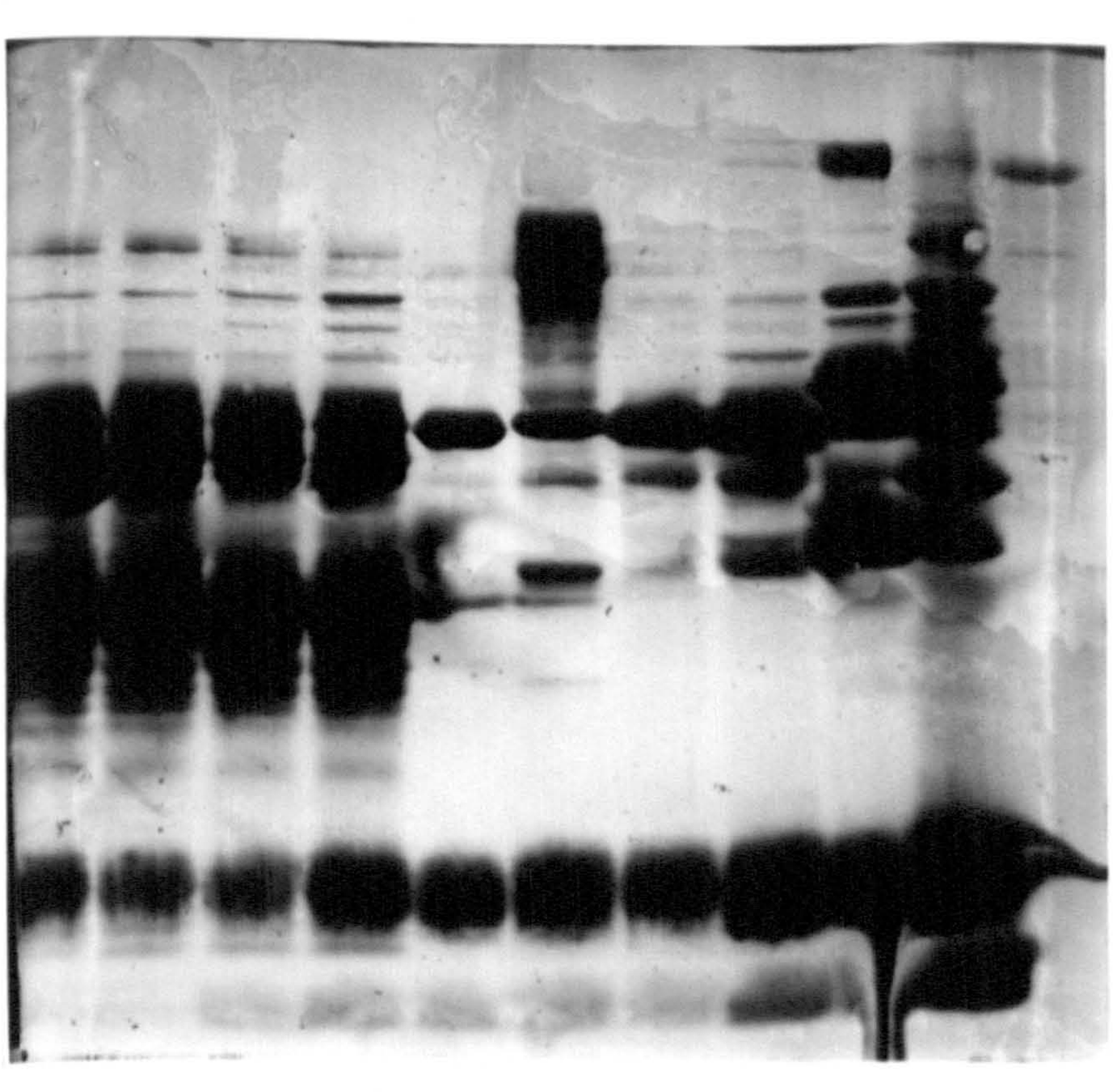


Fig. 4.7. SDS-PAGE of EDTA extracts from 11 Irish isolates (a) stained with Coomassie blue and (b) the corresponding immunoblot.

4.3. Discussion

The immunochemical fingerprinting of C. difficile isolates described here has been used in this laboratory over the past three years to study various outbreaks of disease with which the organism has been associated. The first investigation involved a study of 28 isolates of C. difficile sent from Sweden (Poxton et al., 1984). Some of these were from an outbreak of AAD in a hospital; others from random cases of AAD throughout the country. By use of SDS-PAGE and immunoblotting it was possible to demonstrate the existence of a common strain within the hospital where the outbreak had occurred. The results of this study (in which the isolates had been examined without prior knowledge of their origins) indicated this technique might prove useful in tracing the epidemiology of C. difficile-associated disease.

Since this study questions regarding the level of reproducibilty of the immunoblot patterns produced from one study to the next have arisen. It was noticed that minor variations could occur between blotting patterns produced by different clinical isolates. The assessment of whether these differences were due to real variations between strains or if they arose due to differences in growth conditions or in antigen extraction methods required investigation.

It is apparent from the work done here that the antigenic

nature of any particular isolate of C. difficile will remain constant when analysed by this technique. The only major difference observed with an isolate was when MPRL 589 was left to incubate for an extended period during antigen extraction. It might be that the protein involved was altered by the prolonged exposure to EDTA.

Variations in the amount of protein loaded onto gels (as detected by Coomassie blue staining) can lead to difficulties in interpretation of immunoblot patterns; faintly staining bands often fail to show up properly on immunoblotting. However, provided care is taken, these minor problems do not detract from the usefulness of the technique. Below is a set of guidelines for the successful use of immunoblotting in epidemiological investigations.

4.3.1. Recommendations for successful use of immunoblotting in epidemiological studies

1) Pick several colonies - at least 10 - from the primary isolation plate in case the patient is carrying more than one strain of the organism.

2) A standardised technique should be used for culture and preparation of the EDTA antigen extracts. In this laboratory cells are incubated for 30 min at a temperature of 45°C although minor variations in this do

not appear to matter. Since different strains may vary in their sensitivity to changes during antigen extraction it is best to keep to a well-defined regimen. Freezing and thawing during the EDTA extraction procedure releases more protein: this complicates the Coomassie blue pattern, adds nothing to the immunoblot and should be avoided.

3) Only compare EDTA antigens run on the same gel. It is difficult to compare one gel with another as it is impossible to reproduce running conditions accurately.

4) When analysing SDS-PAGE and immunoblot patterns minor variations should be overlooked if the general picture arising is of similarity between strains.

5) Conclusions should be drawn with information provided by both the Coomassie blue-stained gel and the immunoblot since some patterns that appear similar on the blot can appear quite different on the gel and vice-versa. The advantage of doing an immunoblot as well as a Coomassie blue-stained gel is that the blot is simpler and easier to read.

The Coomassie stain and the immunoblot patterns do vary considerably from one isolate to another although one or two major bands (as well as several minor ones) are

common to all. Some of the major bands staining in Coomassie blue gels are stained by the test antiserum whilst others are not; this is also true for the minor bands. In these studies the variations in staining of the bands can be seen by comparing the patterns produced in Fig. 4.1 and in Fig. 4.6. In Fig. 4.1 the major Coomassie blue-stained bands are also immunogenic, while in Fig. 4.6 only one of the major Coomassie blue-staining bands reacts strongly on the immunoblot and most of the staining on this is due to minor bands. A more detailed study of surface proteins and antigenicity is discussed in Chapter 5.

It would have been interesting to compare the results obtained by use of immunoblotting with other typing techniques. I did attempt to radiolabel all the strains involved in the renal unit investigation with ^{35}S-methionine as described by Tabaqchali et al. (1984). However, although this was repeated several times (including leaving the cells in contact with the radioisotope for 2.5 times the suggested period) there was no incorporation of the radiolabel into the cells. On developing the autoradiograph, only the sample buffer front was visible. This must be attributed to variations in our methodology which did depart from the details in the original manuscript.

Of the methods currently available for typing of C. difficile the most applicable to the diagnostic situation must be either serum agglutination or phage typing. However for both of these to be successful (either diagnostically or in research) a defined battery of sera

would be required or phages isolated. For research purposes, where more time is usually available, immunoblotting appears useful for providing a comparison between isolates. It does not require the use of special facilities (as does radiolabelling) and most laboratories will possess PAGE equipment and blot tanks can be easily constructed in the laboratory. Also, only one antiserum is required for all studies. However, it must be borne in mind that as a consequence of immunoblotting with only a limited selection of antisera, it is not possible to fully 'type' isolates. Immunoblotting used in this manner will only provide a guide to the similarity or otherwise of isolates. It cannot completely characterize them.

The three epidemiological studies described here (as well as several others that I have also been involved in) have provided no definite answer to the question of whether or not C. difficile is an endogenous pathogen or has an environmental source. Unfortunately, no environmental swabs were taken in the MRU during the period of their C. difficile-associated problems. This was a retrospective study and by the time it was undertaken the actual problem had disappeared. The results of the Amsterdam study could point to cross infection between the patients although it is also possible that they both carried this strain normally and both were shedding it into the environment.

The MRU outbreak of C. difficile-associated diarrhoea

appears to have been a true phenomenon as the organism had previously been isolated only very infrequently from this unit. Cross infection was thought to be the likely mode of infection as isolation of patients was limited by lack of space and the specialised nursing that dialysis requires. There were two previous reports of C. difficile being isolated from cases of AAD in renal patients undergoing treatment by either HD or CAPD (Ritchie et al., 1982; Gokal et al., 1982). In both of these cross infection was considered to be very likely although no typing of isolated organisms was undertaken. The five patients from whom the same strain was isolated in our study were probably cross infected; all were nursed in one of two adjacent cubicles, the first four within one month. The isolation of 13 different strains of C. difficile would, however, tend to exclude cross infection as the major mechanism by which C. difficile was acquired during this outbreak.

Fifteen of the 18 MRU patients had received or were receiving antibiotics at the time of C. difficile isolation. Of these, eight had been given cephalosporins. CAPD patients, in particular, tend to be given these broad-spectrum agents since many organisms can cause CAPD peritonitis and treatment often has to be instituted before dialysate culture results are known (Vas, 1983). Renal patients, in general, are prone to infections often requiring treatment with antibiotics. Contributing

factors include relative defects in cellular (Collart et al., 1983) and humoral (Bommer et al., 1983) immunity in uraemia, abnormal nutritional status (Kopple and Jones, 1979), regular cannulation of arterial fistulas and the the presence of foreign bodies (external arteriovenous shunts, peritoneal catheters). On the basis of experience it was recommended to the MRU physicians that it would be best to maintain as narrow a spectrum of antibiotic therapy as possible and to avoid the use of oral antibiotics which could have a more disruptive effect on gut flora than those given by other routes. The use of oral cephalosporins has now been discontinued for peritonitis in the MRU and since April 1984 the rate of isolation of C. difficile from patients has remained low and AAD has not been a major problem.

These findings suggest that cross infection with C. difficile may occur in patients receiving dialysis, although it is not always the major mechanism of acquisition of this organism. It would be unwise to abandon standard measures against cross contamination, and it should be appreciated that patients undergoing dialysis may be particularly prone to infection with C. difficile.

The isolates received from Amsterdam were all found to be similar. This finding was confirmed by both Tabaqchali (with ^{35}S-methionine labelling) and Kuijper (restriction endonuclease analysis of DNA - Kuijper et al., 1987). We were not given many details about the precise origin of the isolates sent from the Dublin hospitals but in the

subsequent correspondence received (from Dr. H. Humphries, St. James's Hospital, Dublin) it seemed that some of the strains may well have been acquired by cross infection.

CHAPTER 5

VIRULENCE FACTORS OF CLOSTRIDIUM DIFFICILE: THE ROLE OF THE BACTERIAL CELL SURFACE

5.1. Introduction

Since C. difficile may be present in the GI tract of persons remaining healthy despite receiving antibacterial therapy, or even in patients who have not received such agents, it seems clear that some additional factor or factors must be necessary for disease to occur. Some of the most obvious candidates for having a potential role in the development of disease symptoms are discussed below.

5.1.1. Levels of C. difficile in the GI tract

Although evidence is conflicting, the severity of disease developing does not appear to correlate purely with the number of organisms present in the gut. Viscidi et al. (1981) found isolation rates of C. difficile from patients with AAD ($10^{4.6}$ per gramme faeces where TCA results were negative and $10^{5.6}$ where TCA was positive) were comparable to isolation rates in antibiotic recipients without diarrhoea ($10^{5.3}$). Nakamura et al. (1981a) found some healthy individuals harboured C. difficile at concentrations about the same or even higher than the mean level of $10^{5.8}$ per gramme faeces reported in patients with PMC (Willey and Bartlett, 1979). The organism can also be isolated from healthy infants at levels between 10^3 and 10^7 per gramme faeces (Stark et al., 1982).

5.1.2. Toxins of C. difficile

Initially, investigators studying the role of toxin in C. difficile-associated disease attempted to correlate cytotoxic activity (as determined by TCA) of what was believed to be a single cytotoxin, with disease severity (Bartlett, 1979; Bolton, 1982). However it is now apparent that all studies published prior to 1981, as well as some since then, were most probably performed with crude extracellular preparations containing a mixture of toxins.

At the present time, four toxins which may be of potential relevance in the pathogenesis of C. difficile-associated disease are known (see section 1.1.3.). Most recent work however, has concentrated on studying two of these toxins, toxins A and B.

5.1.2a Toxin B

This is a potent cytopathic toxin with a cytotoxic activity 1,000 to 10,000 times greater than toxin A (measured on a weight basis with highly purified preparations - Taylor et al., 1981; Sullivan et al., 1982; Banno et al., 1984). Since it is so much more cytotoxic than any of the other toxins and as a consequence of investigators assuming that any strain producing toxin A would always produce B (in the same ratio - Libby and Wilkins, 1982), it has generally been considered that any TCA would detect only toxin B, the

cytopathic effect of A being masked by B.

Haslam et al. (1986) have now shown that the toxins can be produced independently. The relative proportion of toxins A and B produced by any particular strain is very much influenced by the composition of the medium used to culture the organisms. Autoclaving in particular was shown to drastically alter the ability of media to support toxin production. As discussed in section 3.3 this makes the correlation of toxicity values obtained in vitro, with the situation in vivo, practically impossible.

Various investigators did show large differences in the incidence of and cytotoxigenic potential of C. difficile isolates associated with disease (Burdon et al., 1981a; Bartlett and Laughon, 1984; Riley et al., 1986). Bartlett (1981b) found 100% of specimens from 329 patients with AAD or AA-PMC contained cytotoxic activity in TCA and that there were considerable strain variations in the toxic effect produced (Viscidi et al., 1981). Analysis of isolates from cytotoxicity-positive specimens showed a crude correlation between the TCA titres in the stools and those produced in vitro by the strain recovered, an observation not borne out in a similar study by Borriello and Larson (1981). Neither the cytotoxigenic potential of a strain nor the cytotoxic titre in the stool was found to correlate well with severity of clinical disease (Brettle et al., 1982, Burdon et al., 1981b; Trnka and

LaMont, 1984).

A study by Onderdonk et al. (1979) showed 100 to 1000 fold increases in TCA titres obtained when chemostat cultures of C. difficile were subjected to a variety of adverse conditions including an increased Eh (-360mV to +100mV), increased temperature (37°C to 45°C) or the addition of sub-MICs of vancomycin and penicillin. It was speculated that similar alterations in the gut, induced by antibiotics, might prove important in the development of disease.

5.1.2b Toxin A

It has been suggested that toxins A and B are one and the same thing. However, by use of specific antisera to each of the toxins, it has been demonstrated that they are separate entities (Lyerly and Wilkins, 1984; Taylor et al., 1981; Libby and Wilkins, 1982). Since the weak cytotoxic activity of A cannot be neutralized by antiserum specific for B, this effect is considered not to arise due to contamination with B. Also, Lyerly et al. (1986) have examined peptide digest maps of both toxins and no significant homology has been found.

Toxin A may be separated from B by ion-exchange chromatography and further purified by acetic acid precipitation (Sullivan et al., 1982) or PAGE (Taylor et al., 1981). Highly purified preparations (as indicted by a single band on PAGE) have been achieved. This is in

contrast to B where PAGE of the best preparations usually shows multiple bands, each containing cytopathic activity. It may be of course that these are merely different forms of the same toxin (Lyerly et al., 1986).

5.1.2c The potential role of C. difficile toxins in disease

Following discovery of toxin A, investigations to assess the possible role of this in the pathology of disease have been undertaken by many groups. In contrast with Vibrio cholerae and Escherichia coli labile enterotoxins which induce fluid accumulation with no tissue damage (Elliot et al., 1970; Moon et al., 1971), toxin A of C. difficile causes fluid accumulation with mucosal damage in several animal species. These include Syrian hamsters (Bartlett et al., 1978b; Chang et al., 1978; Ebright et al., 1981; Libby et al., 1982), guinea pigs (Rothman, 1981), rabbits (Regh and Pakes, 1982; Banno et al., 1984) and mice (Banno et al., 1981; Taylor et al., 1981; Lyerly et al., 1982). Recently, it has also been reported that treating prairie dogs with cefoxitin resulted in isolation of C. difficile and production of pseudomembranes (Muller et al., 1987). Upon sacrifice, all these animals are invariably found to have haemorrhagic ileocaecitis. The toxin also causes increased vascular permeability in guinea pig skin (Taylor et al., 1981).

A detailed study by Mitchell et al. (1986) provided results that may prove useful in interpreting the clinical syndrome seen in the majority of patients with C. difficile-associated disease. When rabbit ileal and colonic loops were treated with purified toxin A it was found that fluid accumulating in colonic loops contained less protein and was less bloody compared to that in ileal loops. As most patients with C. difficile disease excrete mainly watery stools (few contain blood) this would indicate major colonic involvememt in the disease process. Other patients (with no immediate previous history of diarrhoea) can show evidence of damage to the colonic mucosa. From Mitchell's work (1986) it was shown that tissue damage could occur prior to induction of fluid secretion. This might indicate that patients from whom C. difficile is isolated, who do not have diarrhoea, might still have sub-clinical bowel damage.

This group also found that purified toxin B had no effect on ileal or caecal tissue. Lonnroth and Lange (1983) reported that toxin A on its own caused a clear and watery hypersecretion in mouse intestinal loops but that both A and B were required to produce haemorrhagic hypersecretion in rabbit ileum and hamster caecum. These results are in contrast to the data of other workers (Taylor et al., 1981; Lyerly et al., 1982; Mitchell et al., 1986) who report homogenous toxin A to cause haemorrhagic secretory responses. It might be that these

differences arise due to the use of differing animal models.

It does appear that the continuing belief that toxin B is the major factor involved in C. difficile-associated disease is becoming rather tenuous. Libby et al. (1982) did report that effective protection against clindamycin-induced caecitis was only achieved after immunization against both toxins A and B. Toxin B has been shown to be lethal when given parenterally in several animal species (Arnon et al., 1984; Libby et al., 1982). However the work by Mitchell et al. (1986) and others (Taylor et al., 1981; Lyerly et al., 1982) would indicate protection against A to be sufficient. The need for co-protection shown by Libby et al. (1982) may have arisen since only partially purified toxin B was used. This being the case, the pathogenic effects observed might have been due to very small quantities of toxin A still present in the preparation.

Honda et al. (1983) tested the effects of clindamycin, cephaloridine and tetracycline on the production of toxin A by one C. difficile strain. It was found that clindamycin and cephaloridine stimulated toxin A production by 8 and 16 fold respectively - a very interesting finding considering the established involvement of these agents in disease.

5.1.2d Isolation of cytotoxic C. difficile from healthy infants

About 50% of the C. difficile strains isolated from healthy infants are cytotoxigenic in vitro and crude faecal filtrates give positive TCA results (Chang et al., 1979; Holst et al., 1981; Bolton et al., 1984). Some studies indicate that the cytotoxigenic potential of those isolates is low compared with isolates from symptomatic adults (Bolton et al., 1984) suggesting reduced pathogenicity. However others (Viscidi et al., 1981) have reported no differences between such isolates. It has been suggested that maternal antibody (IgA) secreted in colostrum may provide a protective effect against toxin (George and Hartley, 1983; Kim et al., 1984). George and Hartley (1983) reported that serum antibody titres against cytotoxigenic culture filtrates of C. difficile were high in 8 of 57 (14%) pregnant women compared to non-pregnant individuals. However this is rather a low percentage on which to base any conclusions. Increased faecal cytotoxicity levels have been detected in breast fed infants (Donta and Myers, 1982). It could be that colonisation in infancy is desirable, perhaps inducing subsequent protection via some local immunity. This might explain the low incidence of childhood disease and the variable course of the disease in adults.

It may be that infant gut lacks receptors for C. difficile or its toxins. Cooperstock et al. (1985) suggested that the

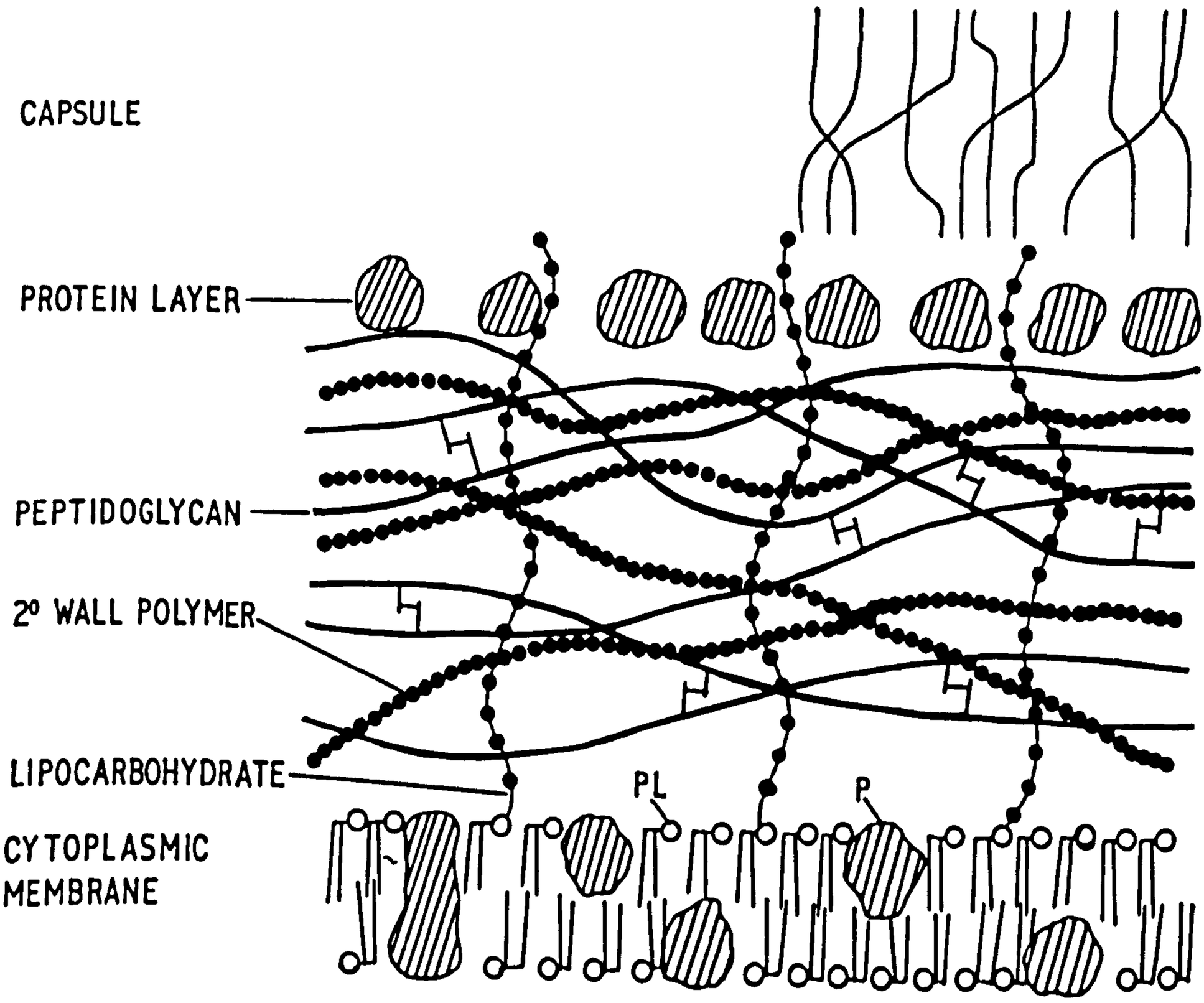
organism might cause subtle mucosal pathological changes that were not clinically apparent. A recent report by Chang et al. (1986) suggested that the apparent resistance of infantile intestine to C. difficile toxins might be related to the nature of intestinal cells rather than intraluminal factors. It was shown that both human amnion (epithelial) and chorion (fibroblasts) cells were relatively insensitive to both toxins A and B during the first week of culture in vitro. As the cell cultures grew older, both types of cells gradually gained susceptibility to the toxins. It was also found that foetal intestinal mucosal tissue did not absorb toxin A or B when exposed, while adult mucosa did so readily.

5.1.3. The bacterial cell surface

To initiate an infection, any potential pathogen requires the ability to associate with particular target cells within the host animal. Having established itself, it will then have to be able to defend against any immune response generated as a result. In fulfilling these requirements, particular components of the bacterial cell surface will be involved to a greater or lesser extent. Very little work has actually been done on the cell surface of C. difficile (or indeed any other Gram-positive anaerobic rod). The basic components that make up the Gram-positive cell envelope are shown schematically in Fig. 5.1. What is known about some of these components in C. difficile is discussed below.

5.1.3a capsule: when observed by phase contrast microscopy in wet india ink C. difficile is seen to possess a small capsule. This contains both carbohydrate and protein in a ratio of 1.0 : 1.8 (Byrne, 1982).

5.1.3b protein layer: Kawata et al. (1984) demonstrated that the cell wall of C. difficile was composed of an inner and an outer wall layer (OWL). This OWL was composed of a regular array of protein subunits, the centre-to-centre spacing between individual subunits being about 8.2nm. This layer projected inwards from the edges of the cell wall and was composed of two main



(Courtesy of Dr. I.R. Poxton)

cross-linking peptide chain

phospholipid

membrane-associated proteins

Fig. 5.1. Schematic representation of the Gram-positive cell envelope.

proteins. Of 11 isolates studied, nine had regular arrays made up of proteins with M_rs of 45 to 47kDa and 32kDa. The other two contained proteins with M_rs of 42kDa and 38kDa.

Similar protein layers have been observed on the walls of other clostridial species where they may be linear (C. novyi, C. tetani, C. botulinum - types A and E), tetragonal (C. sporogenes) or hexagonal (C. thermohydrosulfuricum) in arrangement (Wecke et al., 1974; Takagi et al., 1965; Takumi and Kawata, 1974; Betz and Zeikus, 1968; Sleytr, 1975). Very little is known about the functions of this layer although various suggestions have been made. It could be there as a protective layer against external influences or as a diffusion barrier. It has also been suggested that the layer helps retain toxins within cells (Wecke et al., 1974).

..

5.1.3c cell wall: the basic polymer of the cell wall is peptidoglycan. This consists of parallel polysaccharide chains made up of repeating units of N-acetyl glucosamine and N-acetyl muramic acid which are covalently linked by peptide side chains to form a continuous covalently bound structure around the cytoplasmic membrane and accounts for between 50 to 80% of the Gram-positive cell wall.

The secondary wall polymers, such as teichoic acids and teichoic acid-like polymers have been isolated from the

cell walls of some Gram-positive genera. In many instances these polymers are antigenic and are the species-specific antigens (e.g. in streptococci). They can account for up to 50% of the weight of the cell wall (Baddiley, 1972). There are many variations on the basic structural pattern of these polymers although they were originally defined as polymerised polyol phosphates, often with side chains of mono- or oligo-saccharide units and also ester-linked D-alanine residues (Rogers et al., 1980). Poxton and Cartmill (1982) isolated a carbohydrate-based antigen from C. difficile that appeared to be an analogue of teichoic acid. This consisted of glucose, mannose, galactosamine and phosphate in the approximate molar proportions of 2.0 : 0.65 : 1.0 : 0.63.

Proteins are also present as part of the cell wall but few have been purified and identified. This is because the routinely used methods for purifying cell walls (e.g. boiling 2% SDS, aqueous 80% (w/v) phenol at 0°C) destroy proteins. Milder extraction methods will release only non-covalently bound peptides. Proteases and nucleases can be used to extract covalently bound proteins without denaturation although non-covalently bound material is also likely to be removed.

5.1.3d cytoplasmic membrane: a major membrane polymer of many Gram-positive bacteria is the membrane teichoic

acid, also known as lipoteichoic acid (LTA). This polymer can contribute significantly as a surface antigen. Poxton and Cartmill (1982) extracted such a polymer from disrupted cells of C. difficile by use of phenol. This was found to contain glucose, glucosamine, phosphate and fatty acid in the approximate molar proportions of 2.0 : 1.0 : 1.6 : 0.04. Both this antigen and the teichoic acid analogue showed partial immunological identity and both cross-reacted with C. sordelli antiserum.

5.1.4. The potential role of cell surface components in virulence

a) Motility

Motility has been associated with the virulence of certain bacterial pathogens e.g. V. cholerae(Guentzel and Berry, 1975); Pseudomonas aeruginosa (Holder et al., 1982). From these studies it was found when the motility of these organisms was impaired genetically or immunologically their virulence was reduced. Bacteria that become non-motile or non-chemotactic may have a reduced ability to locate near, adhere to, or penetrate into host tissues; early steps in many disease processes (Savage, 1980; Freter et al., 1978).

When viewed microscopically C. difficile has characteristic motility, especially when grown in CMB. With EM, numerous flagella can be seen covering the surface of the organism; these appendages may well be

important in facilitating association of the bacterial cell with the gut mucosa.

5.1.4b Adhesion

Adherence of microorganisms to surfaces has gained increasing attention as an important initial event in the pathogenesis of bacterial infection; it has been shown to be a crucial step in the production of disease for most of the well-studied enteropathogens (Ofek and Beachey, 1980). This association is essential for at least three reasons: (1) to resist the cleansing action of solutes of the mucosal surfaces, such as mucus in the gut (Attridge and Rowley, 1983); (2) to promote the attachment to target tissues within the host that are distant from the point of entry (e.g. in shigellosis - Labrec et al., 1964) and (3) to deliver toxins in higher concentration to receptors in cell membranes. Attachment has been shown to enhance the virulence of Gram-negative toxigenic enteropathogens such as V. cholerae (Schrank and Verway, 1976), Campylobacter jejuni (Lee et al., 1986) and E. coli (Jones and Rutter, 1972). Most adhesion studies with Gram-positive organisms have been done with staphylococci and streptococci (Shibl, 1985) and have concentrated on the role of teichoic acid and/or LTA (Ofek et al., 1975; Aly et al., 1980; Carruthers and Kabat, 1983).

When discussing bacterial adherence it is vital to differentiate between actual attachment to specific

receptors on cell surfaces and 'association' with, for example, the mucus covering these cells. In the gut this layer can act both as a promotor of attachment or as an inhibitor (Freter, 1982). Borriello and Barclay (1985) claimed to have demonstrated adhesion of C. difficile following recovery of about 10^5 organisms per gramme colonic mucosa (hamster) after extensive washing. However, this study failed to differentiate between true attachment and simple association with mucus. Barer (1984) investigated various methods for studying attachment of C. difficile to various types of cells in aerobic conditions. No evidence for association could be demonstrated when the organism was put in contact with resected human colon or buccal epithelial cells. However, preliminary results with intestinal cell monolayers suggested that some sort of attachment was occurring. It took at least 90 min for significant increases in the number of bacteria associating with cells to occur; thereafter levels continued to rise. It was also suggested that it was important for the cell substrate to be viable but perhaps not so important for bacteria to be viable.

Studies with other Gram-positive bacteria (Streptococcus pyogenes, S. aureus) have indicated that LTA may be implicated in the adhesion of these organisms to cell surfaces (Ofek et al., 1975; Carruthers and Kabat, 1983). Ofek et al. (1975) examined various surface components of

S. pyogenes and found that only LTA was able to inhibit competitively the adhering properties of the organism to any significant degree. Treatment of the organism with diluted antiserum to LTA blocked its binding to epithelial cells. LTA is also thought to contribute to cell surface hydrophobicity (Miorner et al., 1983) It was suggested that LTA molecules on the bacterial surface could allow bacteria to approach the negatively charged epithelial cells, thereby enabling special binding molecules on each of the cell surfaces to interact with each other to form specific bonds of high affinity. Carruthers and Kabat (1983) showed that inhibition by LTA was lost after deacylation of the preparation, perhaps indicating that fatty acid molecules are essential to binding.

A recent study by Wood-Helie et al. (1986) indicated that the surface of C. difficile (at least the nine strains studied) may be hydrophobic. All these strains showed pronounced adhesion to human embryonic intestinal cells and adult colon cells, with no significant difference in the percentage adhesion. It was found that viable bacteria were required for adhesion to both cell lines. Sub-MIC concentrations of antibiotics can affect the adherence properties of microorgansims in various ways (Shibl, 1985). They can inhibit the expression of fimbriae and the synthesis of other surface components; they may also cause the release of cell constituents. The

ability of antibiotics to affect the properties of microbial adherence to cell surfaces may be an important criterion in selecting an antibiotic for therapy; the adherence of some bacteria appears to increase after exposure to antibiotics (Vosbeck et al., 1982). Since the pathogenicity of C. difficile is usually associated with antibacterial therapy this might be a useful area for further studies.

5.1.5. Host defence mechanisms

Variation in immune response to C. difficile infection is another factor that might explain differences in host susceptibilty to disease. Early reports on antibody levels in patients are limited to two case reports where neutralizing serum antibodies to the cytotoxic effects of C. difficile were demonstrated in TCA (Fairweather et al., 1980; Lishman et al., 1981b). Lishman et al. (1981b) reported formation of neutralising antibody in serum from one patient with AAD and also from a healthy control subject. They suggested that the ability of patients to produce neutralising antibodies could affect the severity of disease and might also give protection against further exposure to C. difficile. Nakamura et al. (1981a) found cytotoxicity-neutralising activity in the sera of many young adults and, less commonly, in that of elderly persons. The fact that C. difficile appears to be more common in healthy Japanese adults than in Western

populations (Larson et al., 1978) might account for these differences in antibody formation.

Viscidi et al. (1983) detected toxin A antibody (by ELISA) in 64% of healthy individuals over two years of age and in 19% of those under 2 years. Antibody to toxin B was detected in 66% of those older than six months and in 33% of those less than 6 months. A strongly positive ELISA value for antibody to either toxin correlated closely with the presence of cytotoxicity-neutralizing antibody to the particular toxin being tested (either A or B). Strongly positive ELISA values were obtained more commonly in convalescent sera from 16 patients with C. difficile-induced colitis than in sera from control populations. Testing of paired sera revealed significant increases in the titre of IgG antibody to toxin A or B. There was, however, no evidence for a protective effect in view of the fact that relapse was not uncommon.

Aronsson et al. (1983) did a similar study, investigating antibody levels in AAD and AA-PMC patients. Nineteen of 33 (57%) serum samples from patients with AA-PMC contained antibodies (IgG) to toxin B; one contained only IgA. Twelve of 28 (43%) samples from patients with AAD also contained IgG against toxin B. In neither group was there a response to toxin A. The pronounced difference in antibody levels to toxin A in these two studies is in part due to the fact that Aronsson et al. (1983) determined a base line titre (A_{405} x dilution factor)

equal to 200 for ELISA responses in the control population against which all the other titres were calculated. Thus, their control population showed only 7% of individuals with serum antibody against toxin B and no-one with anti-toxin A even although there had been a response (less than 200) in the ELISA test.

A further study by this group (Aronsson et al., 1985a) has indicated a correlation between clinical recovery without relapse of C. difficile-associated diarrhoea and high IgG titres to toxin B in ELISA and/or neutralising antibodies: 44% of patients had antibody to toxin B and 11% to toxin A. The strong effect of toxin A on the gut as demonstrated by ELISA or neutralising assay determinations during animal experiments (Libby et al., 1982), indicates that it may have a major role in disease. The reported lack of antibody response in serum could reflect low toxin production in the gut, or that there is insufficient absorption from the gut. It may also be that the toxin is only weakly immunogenic in humans. Lack of an IgM response might reflect earlier exposure to the antigen (e.g. in childhood) resulting in a secondary type of response without IgM antibody formation.

5.1.6. Aims of this study:

1. to isolate and study by immunochemical methods-

   a) the carbohydrate molecules associated with both the cell membrane and cell wall of C. difficile

   b) the cell wall proteins

   c) the flagella

2. to assess the potential role of any of these molecules in virulence.

3. to determine the ability of C. difficile to adhere to gut mucosa.

5.2. Results

5.2.1. Immunochemical characterization of the cell surface of C. difficile

a. Studies of the cell membrane carbohydrate antigen

Crude aqueous-phenol-extracted membrane carbohydrate antigen was prepared from four C. difficile strains - NCTC 11223, MPRL 161, RIE 11831 and MPRL 597. Similar extracts were also prepared from C. sordelli NCTC 8780, C. bifermentans NCTC 506 and C. irregularis NCTC 11830. The yield of this material from 6l of culture is shown in Table 5.1.

The C. irregularis had not grown very well when harvested and the carbohydrate extracted from it was found to have a different consistency from all the other preparations which were white and fluffy. The C. irregularis extract was cream in colour and much more powdery.

These crude extracts were separated by SDS-PAGE and stained with silver. The results (Fig. 5.2) show that the patterns produced by the C. difficile strains all appear as regularly spaced ladder patterns. The patterns produced by the other three species were indistinct, and even when twice as much material was applied to the gel no ladder pattern was demonstrated. Immunoblots of the C. difficile extracts probed with antiserum to NCTC 11223 revealed faint patterns of antigenic bands similar to

TABLE 5.1

Yield of membrane carbohydrate obtained from Clostridium species following phenol extraction

Strain		Yield (mg) from 6l of culture
C. difficile	NCTC 11223	34
"	MPRL 161	33
"	RIE 11831	63
"	MPRL 597	55
C. bifermentans	NCTC 506	33
C. sordelli	NCTC 8780	47
C. irregularis	NCTC 11830	6

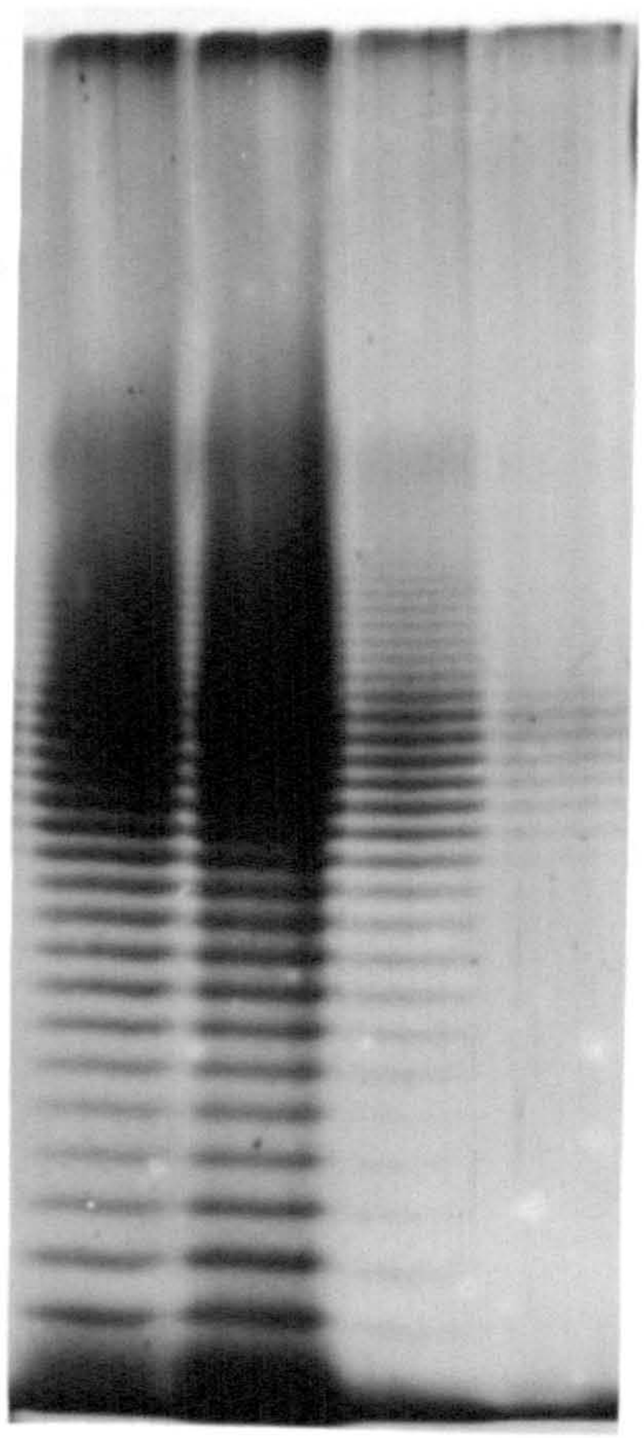


Fig. 5.2. Silver-stained SDS-PAGE of crude aqueous-phenol extracts of C. difficile. Lane 1, NCTC 11223; lane 2 MPRL 161; lane 3 RIE 11831; lane 4 MPRL 597. Samples (50µl) containing 250ug crude antigen were applied to each track.

those of the silver stain. These were too faint to photograph well.

One of these crude extracts (from MPRL 161) was further purified on a Sepharose 6B column. Two antigens were isolated (as detected by FRIE). The first of these eluted in the void volume. This is the volume of fluid required for elution of molecules only distributed in the mobile phase because they are larger than the largest pores in the Sepharose gel. The second antigen eluted with a K_{av} of 0.71. This K_{av} value represents the fraction of the stationary gel volume which is available for diffusion of a given solute species at a particular time.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_e = elution volume of peak
V_o = void volume of column
V_t = total volume of column bed

For the column used here V_e was equal to 20ml; V_o to 8ml and V_t to 9mm x 280mm of Sepharose 6B, i.e. 25ml, thereby giving for the second peak:

$$K_{av} = \frac{20 - 8}{25 - 8} = 0.71$$

The fractions corresponding to the two peaks were pooled, desalted, lyophilized and resuspended in 0.5ml of distilled water before further analysis by SDS-PAGE and immunoblotting. Initial attempts to obtain good photographs of immunoblots were hampered by the apparent lack of sensitivity of the procedure. Subsequently, SDS was omitted from the gels but retained in the sample and electrode buffers. This resulted in greater sensitivity of detection by immunoblotting. The silver-stained gel was very similar to the SDS-containing gel except that the bands ran slower when SDS was omitted. The results obtained by PAGE and immunoblotting of the crude antigen and the two fractions obtained after Sepharose 6B fractionation are shown in Fig. 5.3. The material in fraction 1 gave an identical banding pattern to that seen with the crude material. In the silver stain there was nothing apparent in the sample corresponding to fraction 2. However in the immunoblot an antigenic band was detected in this track just behind the gel front.

Immunoblotting of the C. bifermentans and C. sordelli extracts with antiserum raised against whole cells of C. difficile NCTC 11223 gave a strong cross-reaction in the mid region of the gels. This corresponded to a series of fine lines, appearing as a smear in the silver stain.

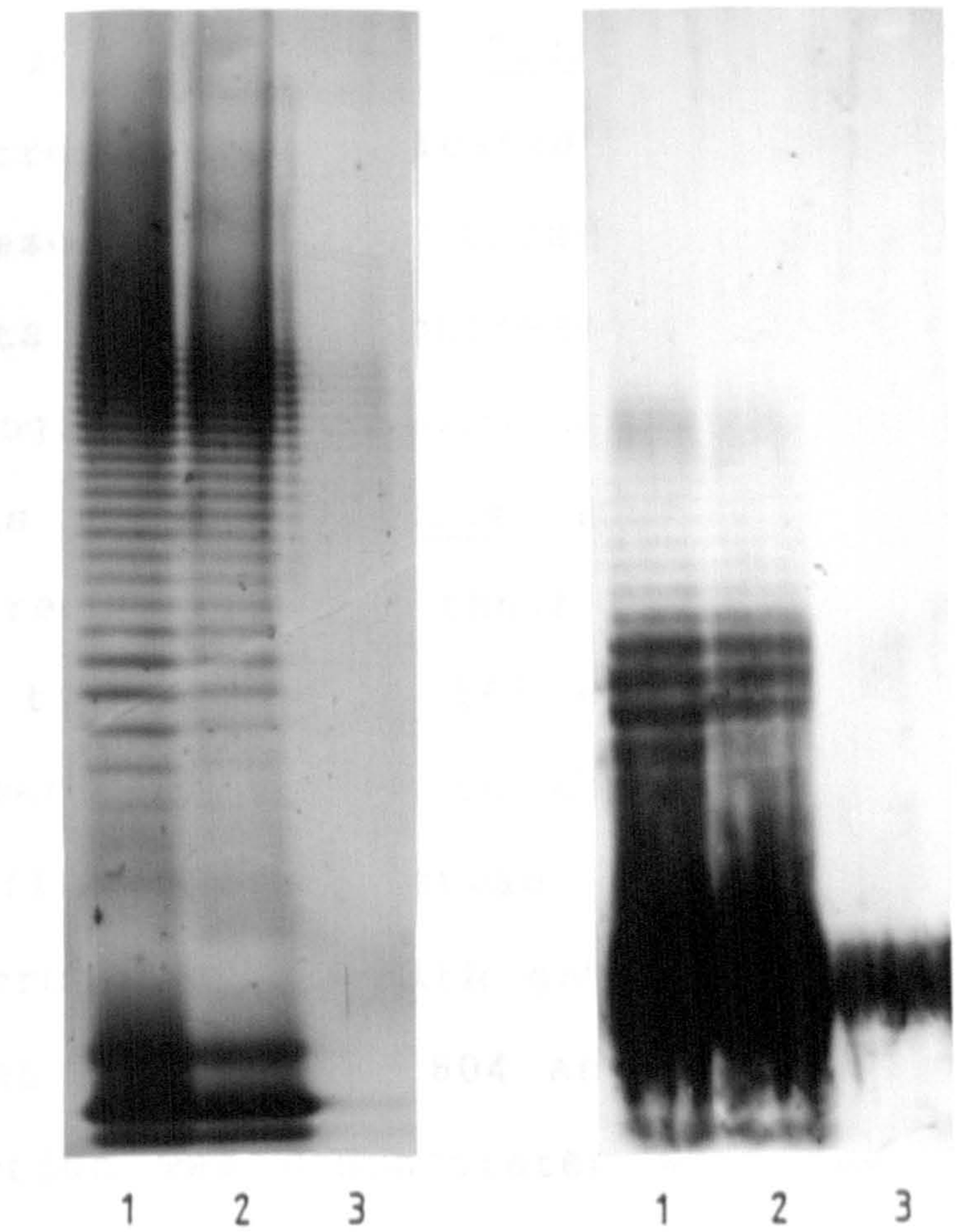


Fig. 5.3. (a) Silver-stained PAGE (without SDS) of crude aqueous-phenol extract of MPRL 161 (lane 1) and the two antigenic fractions detected by FRIE from the Sepharose 6B column (lane 2 is the first antigen and lane 3 the second). Lane 1 contained 250µg crude antigen, lanes 2 and 3 25µg purified antigen.
(b) Corresponding immunoblot with antiserum raised against NCTC 11223 diluted 1 in 250.

5.2.1b Extraction of cell wall carbohydrate

Cell wall carbohydrate was extracted from three strains of C. difficile (NCTC 11223, RIE 11831 and MPRL 161) as detailed in Sections 2.22 and 2.23. Crossed immunoelectrophoresis indicated that there was only one antigen present in each of these preparations (Fig. 5.4). The extracts were electrophoresed (by use of RIE) against both homologous and heterologous antisera raised against whole cells of C. difficile. All three extracts showed antigenic reactions with their homologous antiserum. It was noted that the MPRL 161 extract required twice as much antiserum to focus it compared to the other two extracts (i.e 500µl instead of 250µl). Each of the extracts cross-reacted with antisera raised against RIE 11831, MPRL 11223, MPRL 604 and MPRL 1128. However, no cross-reaction was demonstrated when the extracts were electrophoresed with antisera against MPRL 161, MPRL 683 or MPRL 1123.

Nothing was detected when the three extracts were analysed by SDS-PAGE or were immunoblotted with homologous or heterologous antiserum.

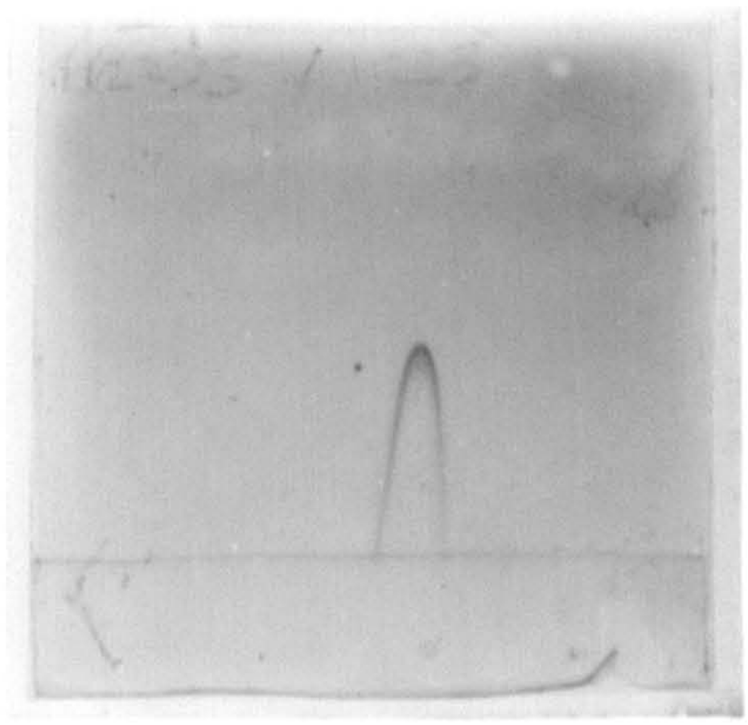


Fig. 5.4. Crossed immunoelectrophoresis of NaOH-soluble cell wall antigen of NCTC 11223 against homologous antiserum (10µl of 5mg/ml solution). Similar pictures were obtained for RIE 11831 and MPRL 161 against their homologous antiserum.

5.2.1c Extraction of cell wall proteins from C. difficile

Crude cell walls from C. difficile NCTC 11223 were prepared as described in section 2.27. This material contained protein and carbohydrate in a 3 : 1 ratio i.e. about 90mg/ml protein to 30mg/ml carbohydrate. Organic phosphate was present at a concentration of about 300µg/ml. After storage (at -20°C) a 1ml sample of the preparation was divided into supernate and pellet by centrifugation and the pellet resuspended in 1ml of distilled water. Protein assays performed on both this and the supernate indicated that up to 48% of the total protein in the preparation was removed in the supernate following repeated freezing and thawing of the extract. Studies done (each with 1ml of crude sample) to find a standard method for extraction of the proteins prior to freezing produced the results noted in Table 5.2.

The heat treated sample was separated into pellet and supernate only after the heating process; all the other methods involved prior centrifugation to obtain the pellet which was then resuspended in 1ml of the test solution. This was subsequently centrifuged to obtain both the supernate and pellet referred to in Table 5.2. Maximum extraction of protein from the pellet was obtained by treatment with 5% SDS, heat or 6M urea. The 6M urea treatment was taken as the method of choice for any further extractions as this was relatively quick

TABLE 5.2

Yields of protein obtained from crude cell wall extracts by use of different extraction techniques

Method of extraction	Protein concentration (µg/ml) Pellet	Supernate	Percentage protein extracted from pellet
Heat (80°C, 10 min)	1871	5537	74.7
2% Triton-X100 (RT, 10 min)	2252	224	9.0
EDTA (10mM, 45°C, 30 min)	898	1018	53.1
Urea (6M, RT, 30min)	455	1186	72.3
Lysozyme (1mg/ml, 37°C, 30 min)	2503	970	27.9
5% SDS (RT, overnight)	461	3539	88.5
1% acetic acid (100°C, 60min)	1030	299	22.5

compared to the SDS treatment and did not involve use of special equipment for heating. Prior treatment with 2% Triton X-100 was also included to remove any cell membrane proteins that might still be present (section 2.28).

When the urea extract prepared from NCTC 11223 was run on SDS-PAGE it was shown to contain three major proteins as well as several minor ones (Fig. 5.5a). The major proteins were calculated to have M_rs of about 46kDa, 32kDa and 28.3kDa. There was also a fairly prominent band at 73kDa. Probing with antiserum raised against whole cells of the homologous strain showed that these four proteins were antigenic (Fig. 5.5b). The three major bands were also apparent on an autoradiograph produced after ^{125}I-surface-radiolabelling of whole cells (Fig. 5.5c). The 73kDa antigen did not show up on this.

Urea extracts were prepared from a further four strains of C. difficile (MPRL 604, MPRL 683, MPRL 1123 and MPRL 1128), all of which had been shown to be different by prior SDS-PAGE and immunoblotting of EDTA extracts. When run on SDS-PAGE these extracts were all found to contain between one and three major protein bands, all having different M_r values (Fig. 5.6 and Table 5.3). In some instances it was difficult to assess whether the bands in the different isolates represented similar or different proteins as they were very thick. Molecular mass

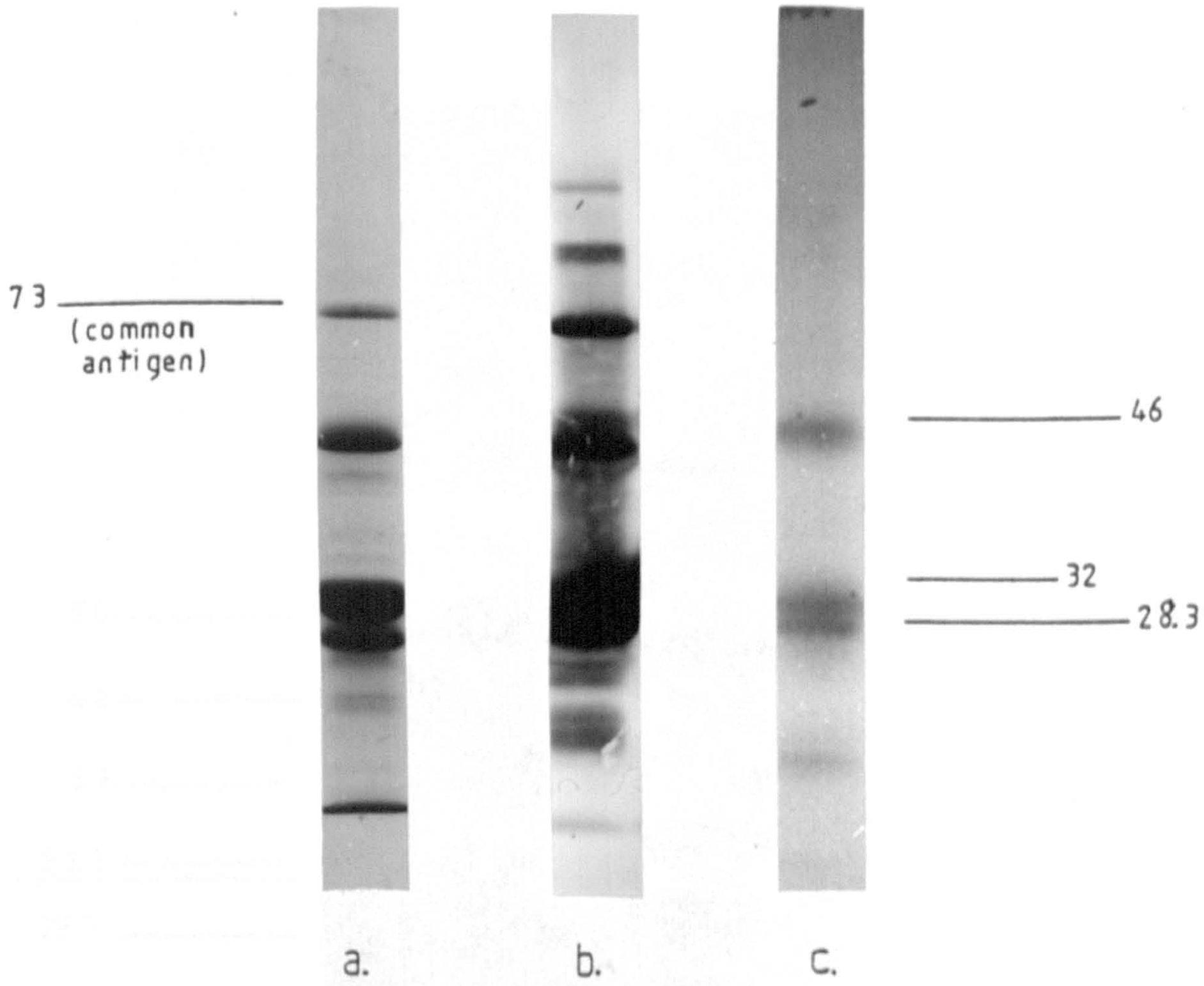


Fig. 5.5. SDS-PAGE (a) and immunoblot (b) of urea extracted proteins from NCTC 11223; (c) is an autoradiograph of whole cells radiolabelled with I^{125}.

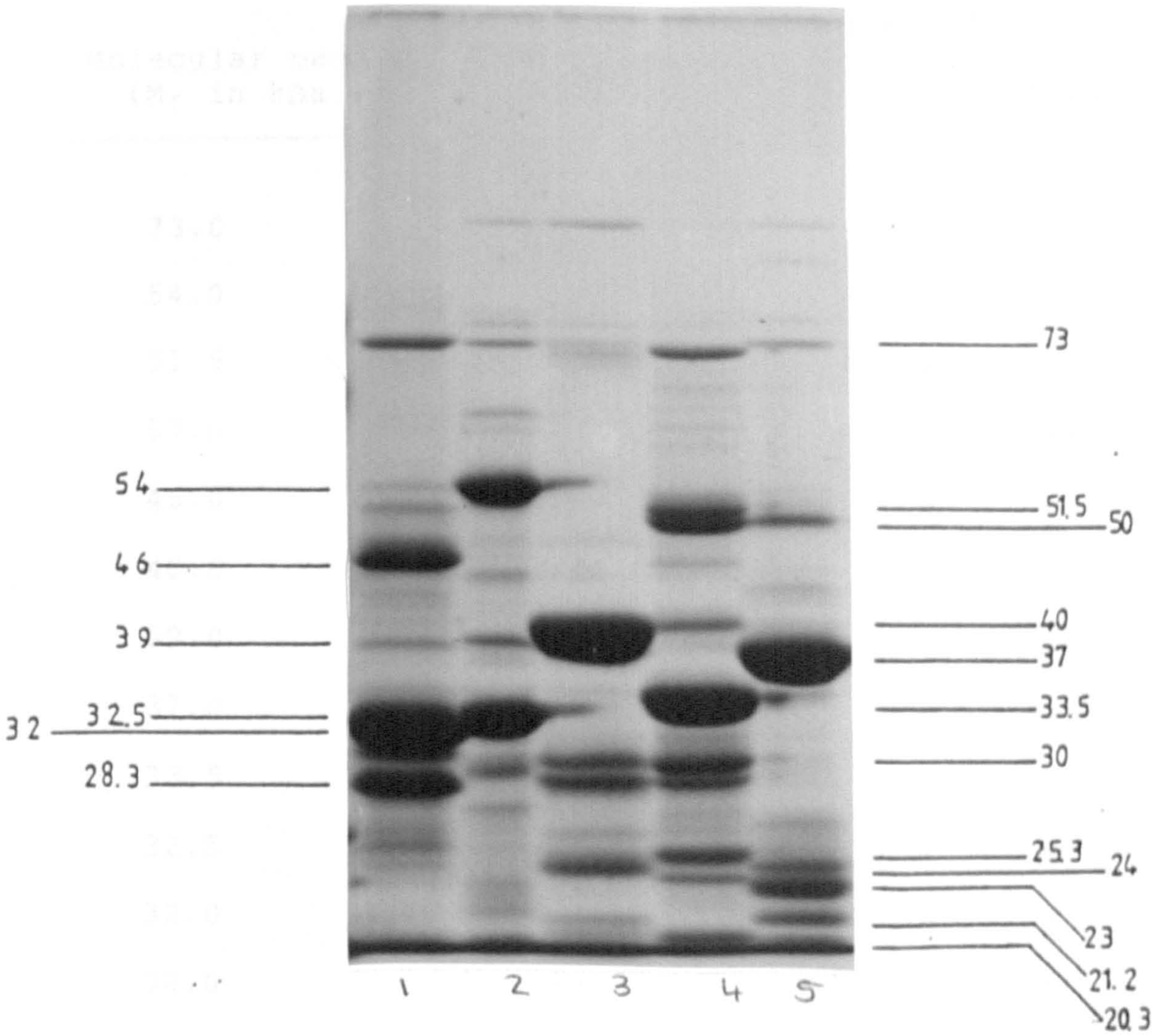


Fig. 5.6. SDS-PAGE of cell wall proteins extracted from C. difficile with 6M urea. Track 1, NCTC 11223; track 2, MPRL 604; track 3, MPRL 683; track 4, MPRL 1123; track 5, MPRL 1128.

TABLE 5.3

Protein bands separated from strains of C. difficile following SDS-PAGE of urea exracts of cell walls

Molecular mass* (M_r in kDa)	Strain** NCTC 11223	MPRL 604	MPRL 683	MPRL 1123	MPRL 1128
73.0	++	+	+	++	+
54.0	+	+++	-	-	-
51.5	-	-	-	++	-
50.0	+	-	-	++	++
46.0	+++	-	-	+	-
40.0	-	-	-	+	-
39.0	+	+	+++	-	-
37.0	-	-	-	-	+++
33.5	-	-	-	+++	-
32.5	-	+++	-	-	-
32.0	+++	-	-	-	-
30.0	++	+	++	++	+
28.3	+++	-	++	++	-
25.3	+	-	-	++	-
24.0	+	-	++	-	+
23.0	-	-	-	+	++
21.2	-	-	+	-	++
20.3	-	-	-	+	+

see over/

* : molecular mass was determined by measuring the distance travelled by a protein band in Fig. 5.5 and converting this by use of the standard curve of log M_r against percentage mobility in SDS-PAGE in Appendix 4.

** : intensity of the bands was recorded as +++ : strongly staining band, ++ : moderately staining band, + : faintly staining band, - : no band of this M_r.

measurements were in fact made from the centre of all the bands. I did suspect that the proteins with M_rs measured to be 32.5kDa and 32kDa (in the extracts from MPRL 604 and NCTC 11223 respectively) might in fact be the same protein. However, there was no cross-reaction when either extract was probed with antiserum raised against whole cells of the other strain.

There were two protein bands on Coomassie staining that were common to all the strains (Fig. 5.6); one with an M_r of 73kDa and the other with M_r of 30kDa. Although the 73kDa band was faint in the Coomassie stains of some of the extracts it always gave a very strong antigenic response when each strain was probed both with homologous antiserum and with antiserum raised against the other four strains. Also, when probed with homologous antiserum that had been absorbed with whole cells of the other strains, the staining of the band decreased. This would indicate that this particular protein was a common antigen. The other band (30kDa) was only antigenic (when tested with homologous antiserum) in MPRL 604, MPRL 1128 and possibly in NCTC 11223. The immunoblot of this latter strain was too complex to be certain about whether one very fine band was giving a response. There was no cross-reaction of these proteins when immunoblots were probed with heterologous antiserum. Therefore it would appear that these are different proteins even although they all have the same M_r value.

All of the major proteins (+++ in Table 5.3) were shown to be antigenic when probed with homologous antiserum (Figs 5.5 and 5.7i to iv). Most of these proteins appeared to be unique to particular strains but minor bands having the same M_r values were present in some of the other strains. The 46kDa antigen demonstrated with homologous antiserum present in MPRL 1123 appeared to be the same antigen as the major protein in NCTC 11223. There was apparent cross-reaction when probed with antiserum against NCTC 11223 but the reaction did not diminish with use of absorbed homologous antiserum. The converse reaction i.e. reacting the NCTC 11223 extract against antiserum to MPRL 683 did not occur. This shows that although these proteins had the same M_r they were different antigens.

Protein bands of 28.3kDa were present in MPRL 683, MPRL 1123 and NCTC 11223. In NCTC 11223 this was a major antigen but in MPRL 1123 and MPRL 683 the protein was not antigenic when probed with homologous antisera. However the MPRL 683 extract cross-reacted with antiserum against NCTC 11223. This reaction was not abolished when absorbed NCTC 11223 antiserum was used. The converse reaction produced no response. It may be that this protein occurs in MPRL 683 at levels too low to elicit a strong antibody response in the rabbits. However, when probed with heterologous antiserum containing high levels of antibodies to the protein a reaction could occur.

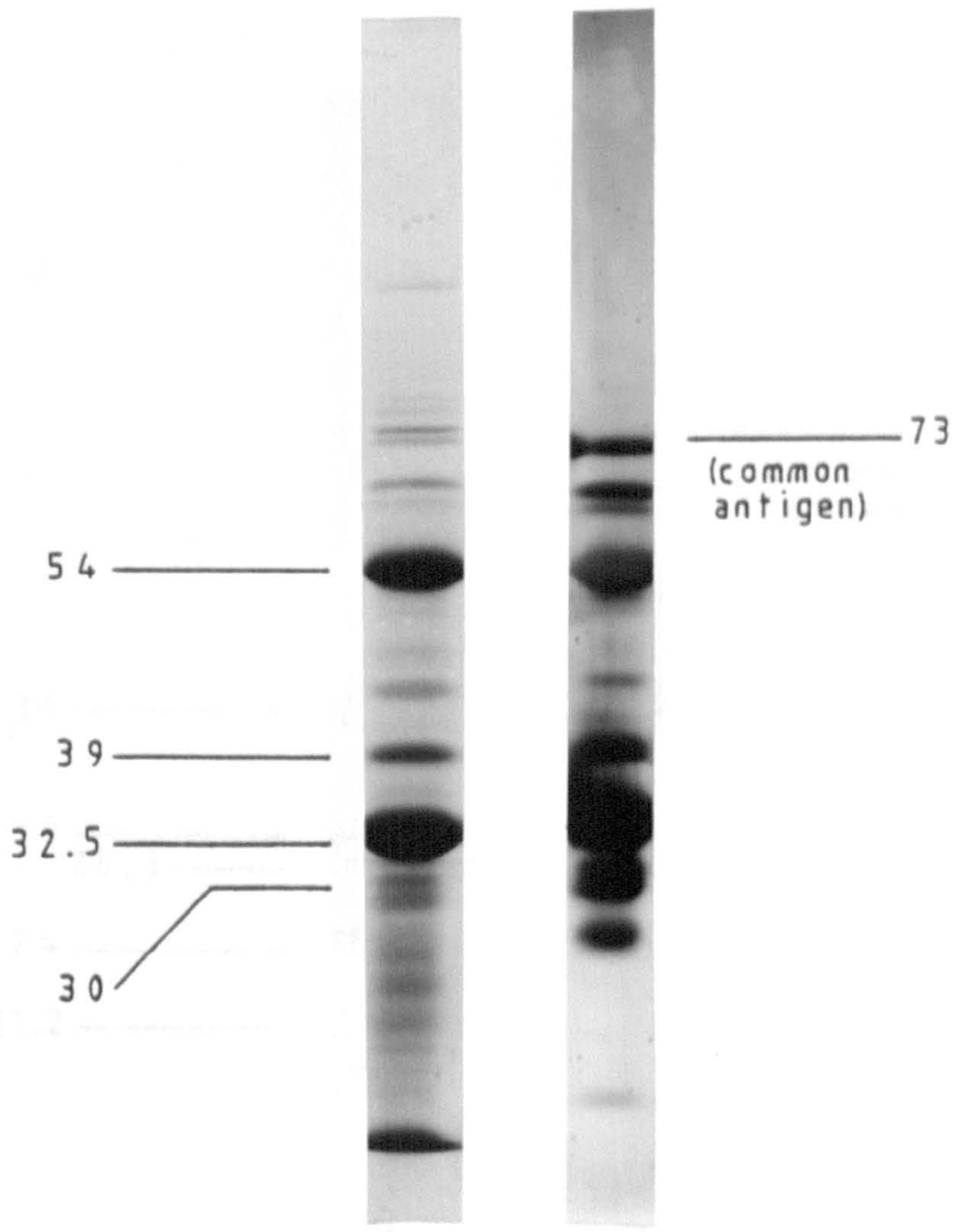


Fig. 5.7. (i) SDS-PAGE (a) and immunoblot (b) of urea extracted proteins from MPRL 604 probed with homologous antiserum.

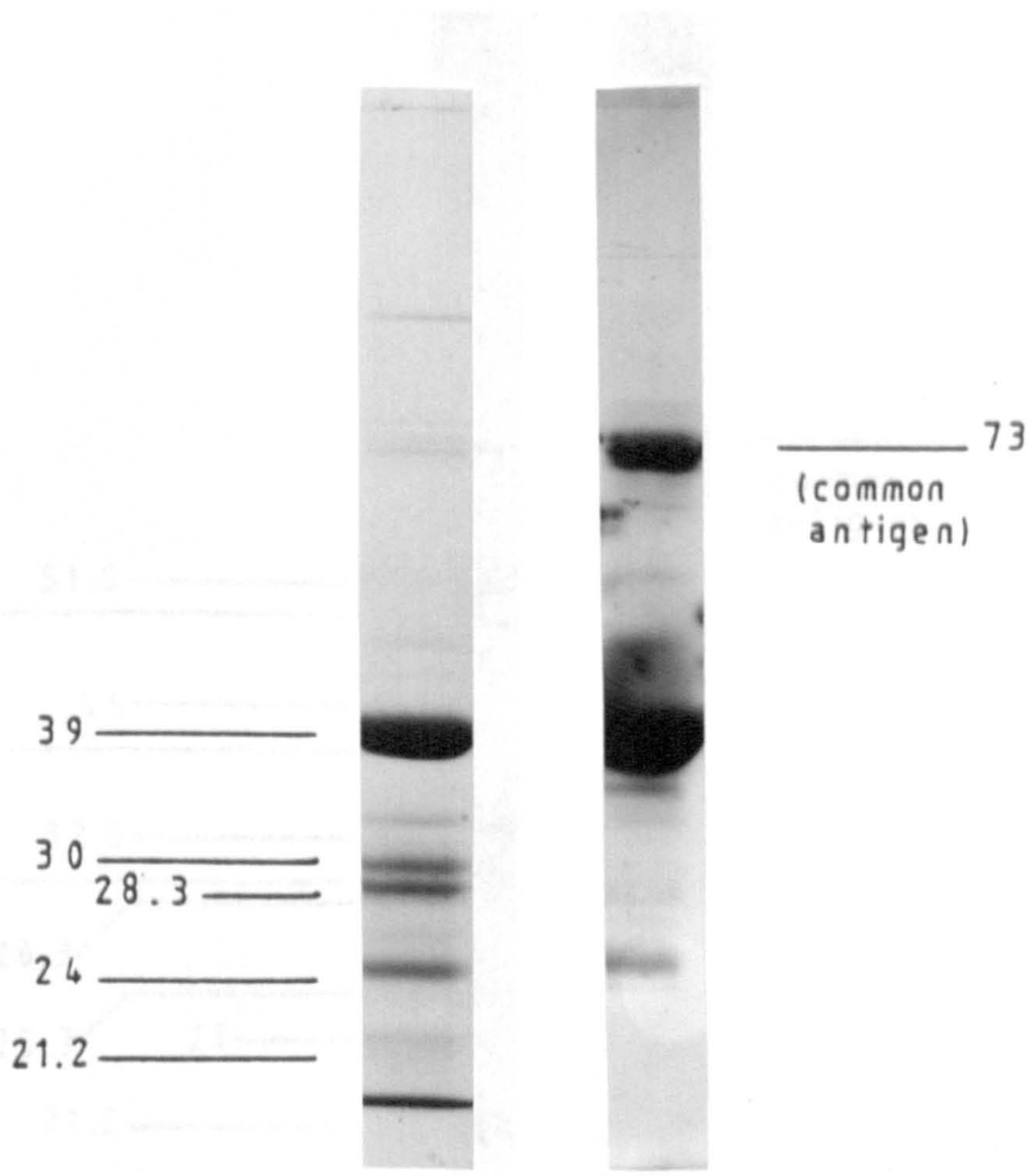


Fig. 5.7. (ii) SDS-PAGE (a) and immunoblot (b) of urea extracted proteins from MPRL 683.

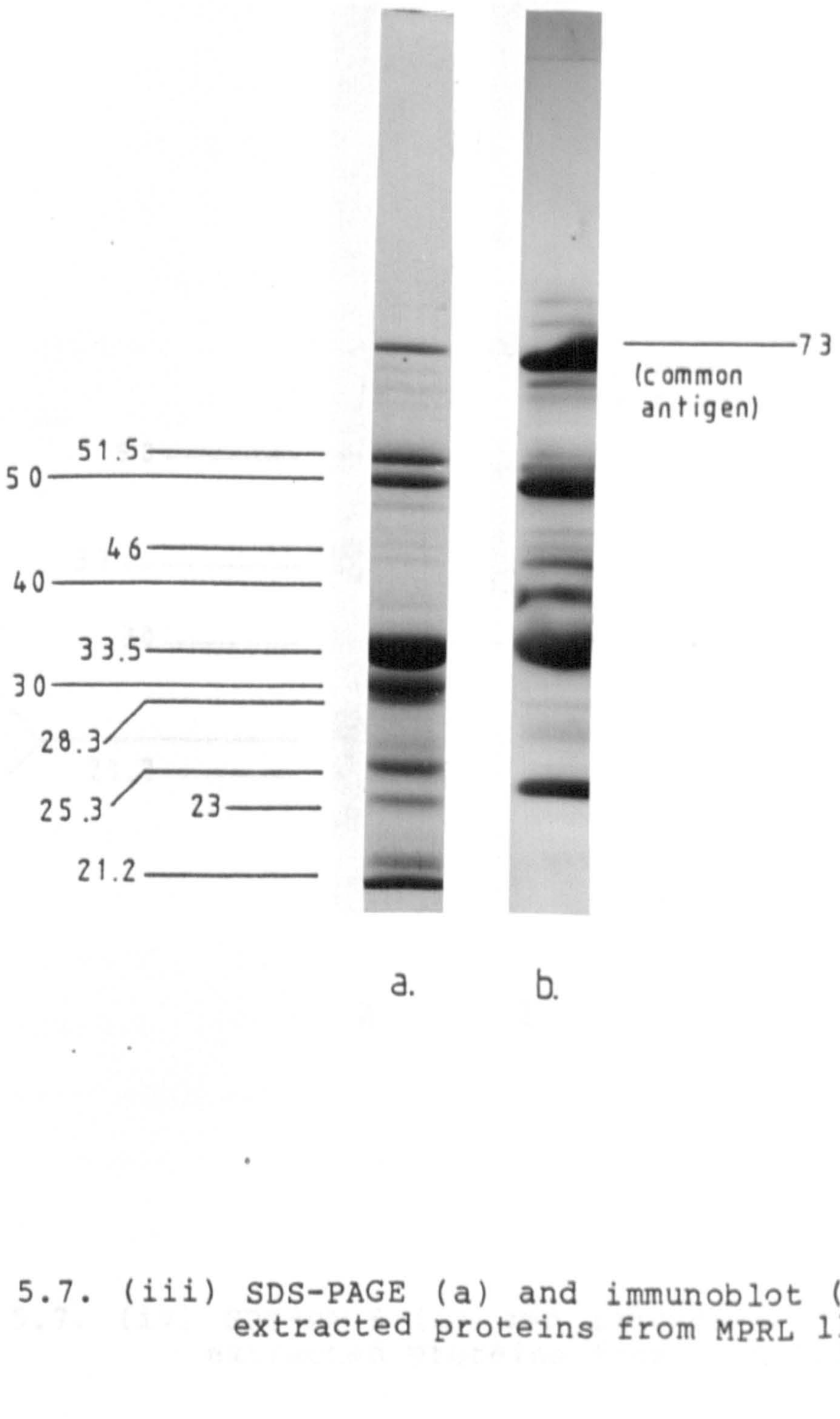


Fig. 5.7. (iii) SDS-PAGE (a) and immunoblot (b) of urea extracted proteins from MPRL 1123.

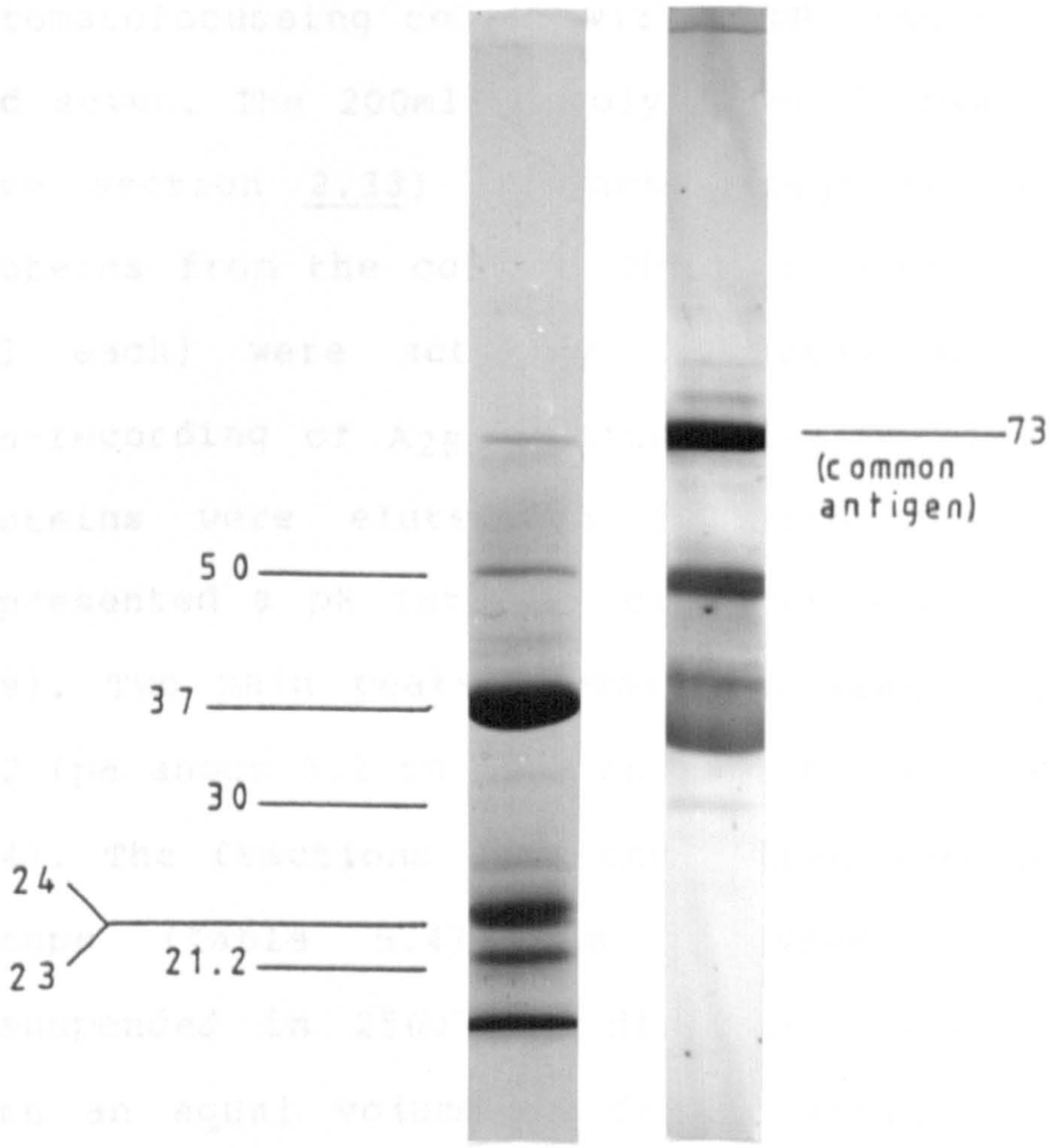


Fig. 5.7. (iv) SDS-PAGE (a) and immunoblot (b) of urea extracted proteins from MPRL 1128.

Iso-electric focussing of the urea extract of NCTC 11223 indicated that all the proteins had pI values of between five and three. The extract was subsequently applied to a Chromatofocussing column with a pH gradient between four and seven. The 200ml of Polybuffer 74 made up originally (see section 2.33) was not enough to remove all the proteins from the column. Three hundred fractions (about 1ml each) were actually collected and a continuous pen-recording of A_{280} against time obtained (Fig. 5.8). Proteins were eluted in fractions 70 to 210. This represented a pH interval of about 5.5 to 4.1 (see Fig. 5.9). Two main peaks appeared between fractions 98 and 112 (pH about 5.1 to 5.2) and 181 to 189 (pH about 4.3 to 4.4). The fractions from the column were pooled into 12 groups (Table 5.4) and dialysed, lyophilised and resuspended in 250ul of distilled water. After mixing with an equal volume of double strength sample buffer 50ul of each of the 12 fractions was applied to a gel. It was apparent from this gel that the proteins had not been very well separated on the column. The protein with M_r of 73kDa was not apparent on the gel while the proteins with M_rs of 46kDa, 32kDa and 28.3kDa were spread over pooled fractions 8 to 12 which were at pHs ranging from 4.1 to 4.6.

It is clear that the surface proteins of this particular strain of C. difficile are highly charged. In order to assess the nature of the charges occurring on the cell surface of the organism as a whole, hydrophobicity assays

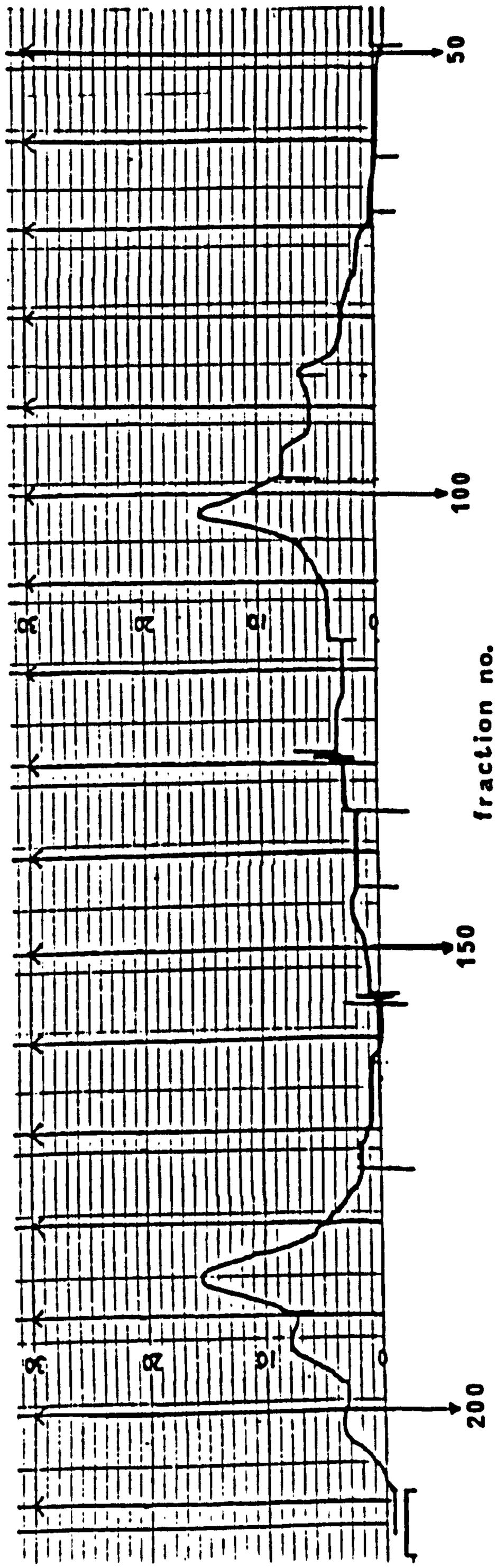


Fig. 5.8. Pen-recording of A280 produced by Chromato-focussing column eluent passing through a flow cell in a Pye Unicam SP6 spectrophotometer. Column was composed of Polybuffer exchanger PBE 94.

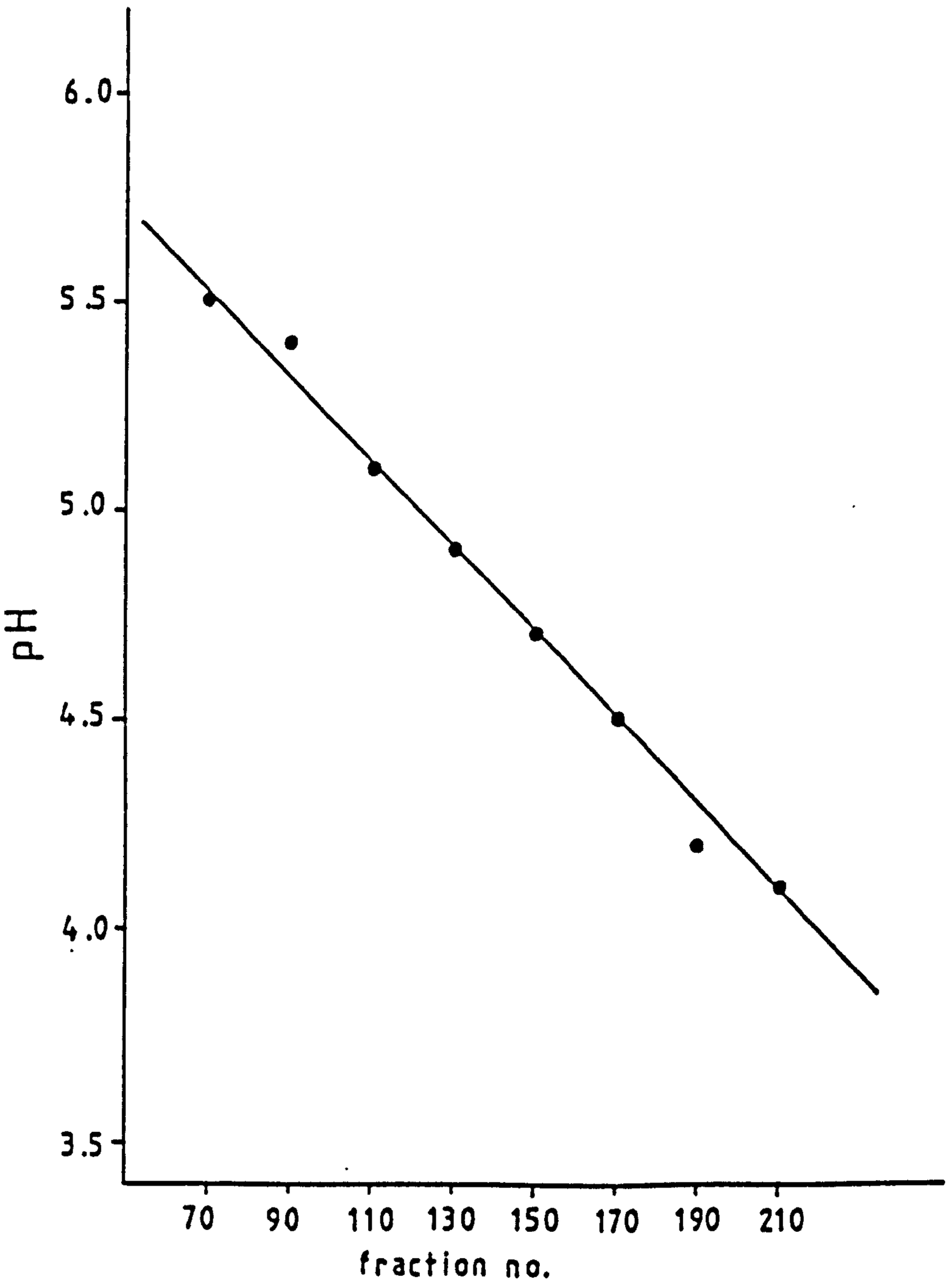


Fig. 5.9. pH levels measured in fractions collected from the Chromatofocusing column. Column was equilibrated between pH 7.0 and pH 4.0.

TABLE 5.4

Fractions pooled following Chromatofocussing of urea extracted cell wall proteins from C. difficile NCTC 11223

Fractions off column	Tube Nos.*	Pooled fraction number
68 - 76	69 - 77	1
77 - 92	78 - 93	2
93 - 97	94 - 98	3
98 - 112	99 - 113	4
128 - 130	129 - 131	5
144 - 151	145 - 152	6
154 - 156	155 - 157	7
163 - 172	164 - 173	8
175 - 180	176 - 181	9
181 - 189	182 - 190	10
190 - 198	191 - 199	11
199 - 209	200 - 210	12

*: there was a time lag of 2 min 45 sec between a sample passing through the spectrophotometer and its eventual collection by the fraction collector. As the collector was set to move once every three minutes this meant it was operating about one fraction behind the spectrophotometer.

were performed on NCTC 11223 as well as several other isolates. The particular assay system used (see section 2.7) measured the partition of strains into octane compared to buffer solution. Hydrophobic strains would be expected to partition into the hydrocarbon (resulting in a large decrease in OD of the aqueous phase) while hydrophilic strains would remain in the aqueous buffer phase. The results of these assays (following culture of the organisms in PPY medium) are in Table 5.5. All were done in triplicate (at least). A S.aureus strain was included in the assay as this species is known to be hydrophobic (Rosenberg et al., 1980). It can be seen from the results that nearly all the strains tested appear to be fairly hydrophilic by comparison. Only two isolates (MPRL 604 and MPRL 678) had a decrease in OD greater than 60%. Both these isolates were in fact shown to be the same strain by SDS-PAGE and immunoblotting of EDTA extracts.

This assay was repeated with the five strains from which urea extracts had been prepared. The technique employed for the assay was identical except that the organisms were cultured for 24h on BA instead of in PPY medium prior to testing. There was no change in the percentage decrease values obtained for MPRL 683, MPRL 1128 and NCTC 11223. The decrease with MPRL 604 was reduced to about 50% while the results for MPRL 1123 were more consistent than previously achieved, with values between 22% and 33%.

TABLE 5.5

Results of hydrophobicity assays on C. difficile after culture in PPY medium

Isolate	Percentage decrease in optical density			Average
MPRL 1122	9	11	13	11
MPRL 1124	15	13	13	14
MPRL 1121	15	12	15	14
NCTC 11223	15	20	10	15
MPRL 1128	17	18	17	17
MPRL 715	22	22	16	20
MPRL 683	22	22	22	22
MPRL 161	21	28	37	29
MPRL 718	29	38	28	32
RIE 11831	33	32	32	32
MPRL 678	73	70	69	71
MPRL 604	71	69	75	72
MPRL 1123	48	56	100	*
Staphylococcus aureus PR1	87	85	83	85

* : results produced by this strain were not reproducible ranging from 100 to 48%

5.2.1d Isolation of flagella from C. difficile

When viewed microscopically, especially following culture in CMB, nearly all strains of C. difficile are motile. There are, however, clear differences in the intensity of the movements observed between strains. Slide agglutination of three formalinised strains of the organism, done with homologous antiserum (see sections 2.10 and 2.11 - Widal test), gave positive results and cross-reaction was also demonstrated (Table 5.6). In many species such reactions involve the flagella present on the bacterial cell surface. Consequently, it was decided to investigate these appendages more thoroughly.

Attempts were made to prepare flagella from four strains of C. difficile; RIE 11831 and MPRL 161 which demonstrate very active motility, NCTC 11223 which is only sluggishly motile and MPRL 558 which grows mainly as elongated cells showing little movement. After final purification with caesium chloride equilibrium-density-gradient centrifugation these preparations were viewed with an inverted microscope to check the position of any bands present. RIE 11831 had two clearly distinguishable bands lying about a quarter of the way down the centrifuge tube while MPRL 161 had one thick band lying in an equivalent position (Fig. 5.10). There were no bands apparent in the MPRL 558 preparation and there was only one very faint band in the NCTC 11223 preparation. This was in the same position as the upper band from RIE 11831.

TABLE 5.6

Slide agglutination of formalinised C. difficile cells against homologous and heterologous sera

Strain	Motility*	Agglutination with antiserum against		
		NCTC 11223	MPRL 161	RIE 11831
NCTC 11223	+	+	-	-
MPRL 161	+++	-	+	+
RIE 11831	+++	-	+	+

*: motility was assessed on a comparative basis after 24h incubation of cells in CMB. +++ : very actively motile; + : only sluggish motility

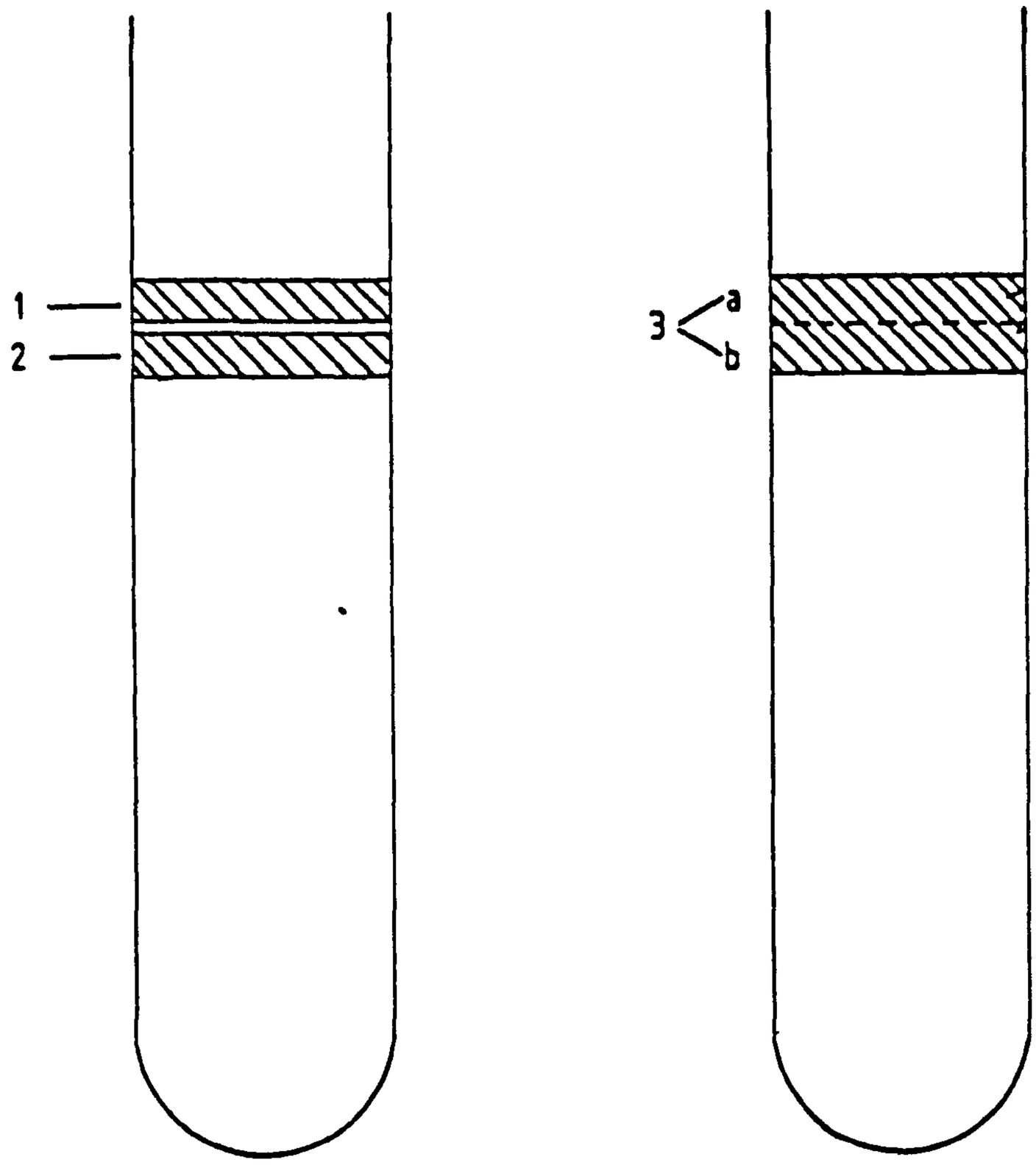


Fig. 5.10 Bands obtained following caesium chloride equilibrium-density-gradient centrifugation of crude flagellar preparations from RIE 11831 and MPRL 161. Bands 1 and 2 were collected separately; band 3 was collected as two fractions a and b.

The two bands from RIE 11831 were collected separately (1 and 2). The RI of both bands was checked. The upper one had a RI of 1.3625; the lower one was 1.3640. These values are equivalent to densities of 1.295g/cm^3 and 1.310g/cm^3 respectively. An upper and a lower fraction were collected from the thick band present in MPRL 161 (3a and 3b); the NCTC 11223 and MPRL 558 preparations were discarded. After removal of the $CsCl_2$ present in the samples (see section 2.20) each was applied to a Formvar-coated copper grid, gold-palladium shadowed and viewed by EM.

Flagella were clearly seen in three of the four fractions (Table 5.7). There were many of the appendages present in both fractions collected from MPRL 161 (see Fig. 5.11). It was noted that they were not evenly spread over the surface of the grid and in both samples they appeared closely associated with large deposits of material. These may well have been aggregations of flagella that had not been dissociated during the resuspending process. There appeared to be less flagella present in band 3b but this may have been an artifact produced during preparation of the grid. Band 1 from RIE 11831 gave the same sort of picture but there were fewer of the appendages apparent. Band 2 appeared to have no flagella present. There was also a lack of any of the clumpy material seen with the other fractions.

Protein assays of the preparations did not provide

TABLE 5.7

Relative proportions of flagella seen in fractions harvested from a $CsCl_2$ gradient viewed by electron microscopy

Strain	Band	Flagella*
RIE 11831	1	++
" "	2	-
MPRL 161	3a	+++
" "	3b	++

*: number of flagella present were graded +++ to + on a purely comparative scale. -: no flagella present

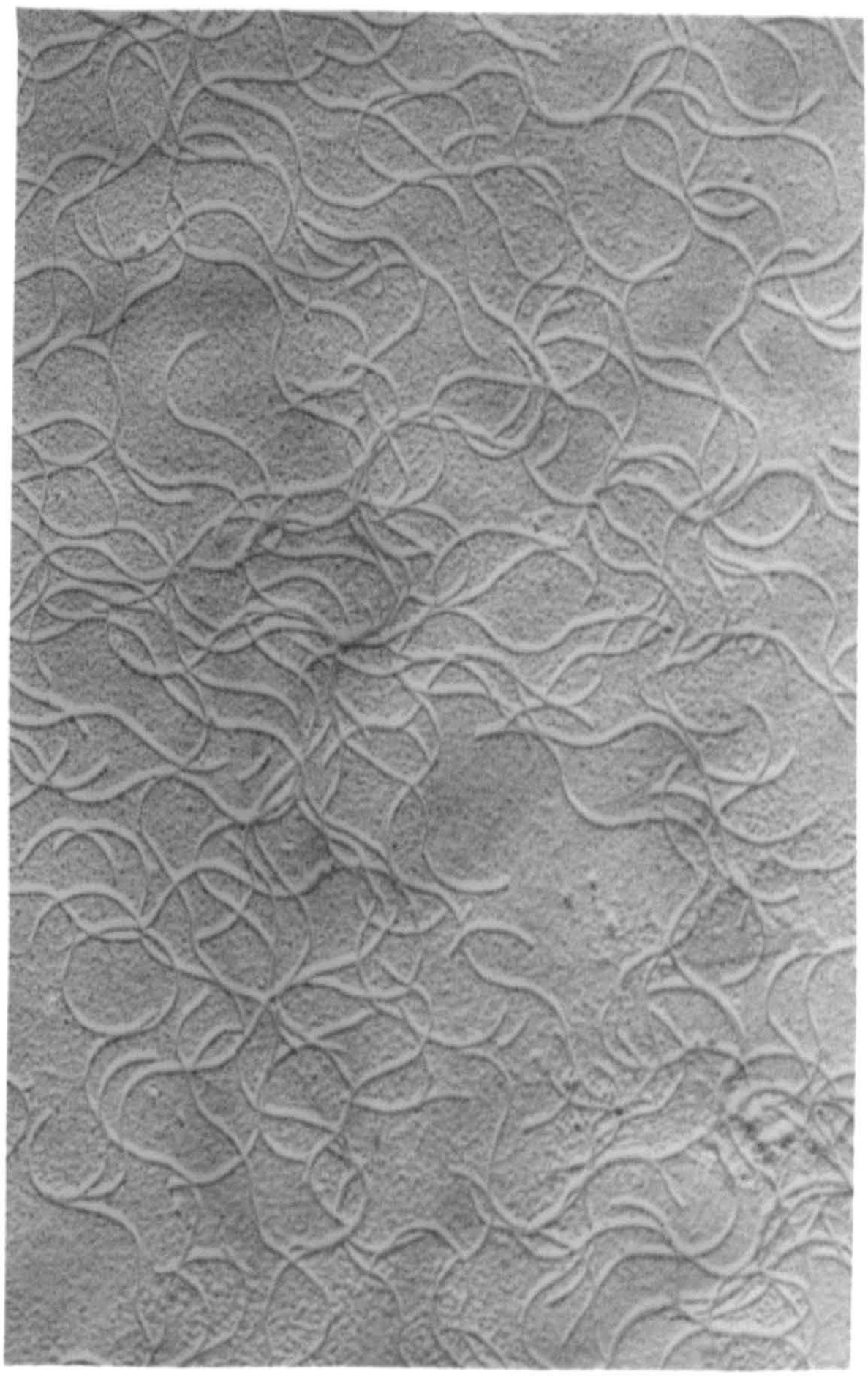


Fig. 5.11 Flagella (magnified 27,000 times) from MPRL 161 (band 3a) as seen by electron microscopy following gold-palladium shadowing.

detectable results. However, after mixing each fraction with an equal volume of double strength sample buffer and boiling for 10 min, 50µl of this was loaded onto a gel for SDS-PAGE and immunoblotting. Broad protein bands were apparent in the Coomassie stain of fractions 1 and 2 from RIE 11831 (Fig. 5.12). Much fainter bands were present in samples 3a and 3b from MPRL 161. Each band was probed in an immunoblot with antiserum raised against the homologous bacterial strain. There was a strong antigenic reaction with both fractions from each of the strains. When tested against antiserum raised to the other strain cross-reaction was demonstrated. Reaction with antisera raised against MPRL 604, MPRL 683, MPRL 1123 and MPRL 1128 also gave a very weak positive immunoblot response. However when the preparations were tested against antiserum to NCTC 11223 there was no reaction at all.

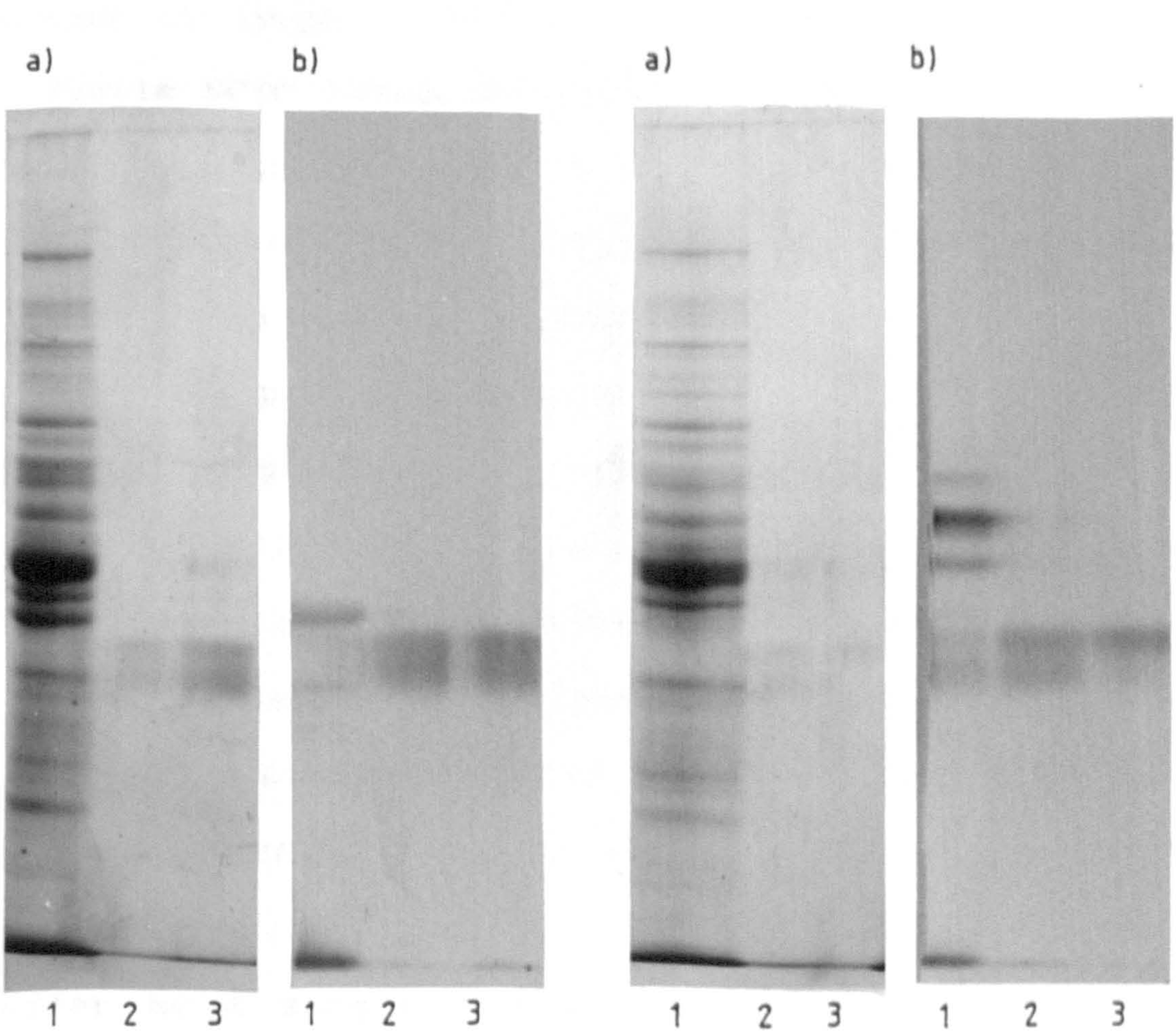


Fig. 5.12 SDS-PAGE (a) and immunoblot (b) of flagella from RIE 11831 and MPRL 161. Track 1 contains an EDTA extract of each strain. Track 2 contains the upper band or fraction from the $CsCl_2$ gradient and track 3 the lower band or fraction.

5.3. Discussion

5.3.1. The carbohydrate antigens of C. difficile

Previous work in this laboratory had shown that a phenol extract of lyophilized supernate from broken cells of C. difficile NCTC 11223 contained two antigens when studied by CIE (Poxton and Cartmill, 1982). These antigens, which appeared to be antigenically related, were proposed to correspond to a) the LTA moiety, a type of which is found in all Gram-positive organisms and b) a deacylated form of this antigen which was found to cross-react with the cell wall antigen (teichoic acid).

The LTA of most Gram-positive organisms has been reported to be based on a poly-(glycerol phosphate) backbone. It might be expected that such a molecule would not show a ladder pattern such as was detected here when analysed by PAGE. If the chain lengths were variable they would only differ by a single substituted glycerol phosphate unit which would probably not be resolved by PAGE. In C. difficile the fatty acid antigen appears to be of a more complex nature. It seems to be in a form more akin to the smooth lipopolysaccharide of Gram-negative bacteria which also produces a ladder pattern when examined by PAGE (Tsai and Frasch, 1982). As such, it may be that the molecule is composed of polysaccharide units, each differing by one oligosaccharide repeating unit.

All immunoblots were performed with antiserum raised

against C. difficile NCTC 11223. The four crude phenol extracts examined all produced the same ladder pattern of reactivity. It seems therefore, that this is a cross-reactive antigen present in all the C. difficile strains examined.

As far as I am aware this is the first report of the analysis of a LTA (or its analogue) by PAGE. It has however been speculated that a ladder pattern seen in immunoblots of crude cell surface extracts of C. botulinum might be due to such an LTA-type molecule (Poxton, 1984). I do not know if this ladder pattern would be produced by the LTA of many other clostridia. Silver-stained gels of the C. sordelli and C. bifermentans extracts did not give this pattern although there was antigenic cross-reactivity when the carbohydrate was probed with the antiserum raised against C. difficile NCTC 11223.

The identity of the second antigenic peak from the Sepharose 6B column remains uncertain. This rather heterogenous low-M_r material might be fragmented wall antigen or deacylated membrane antigen. purified wall carbohydrate could not be detected by SDS-PAGE which may indicate the second antigen was more likely to be deacylated membrane carbohydrate. However, since native wall antigen may be too large to enter the polyacrylamide gel this may have accounted for the results observed.

The sodium hydroxide-extracted wall antigen obtained from

three species of C. difficile all reacted with their homologous antiserum. However none of them cross-reacted with three of the sera tested (i.e. that raised against MPRL 161, MPRL 683 or MPRL 1123). It was noted that the extract of MPRL 161 required twice as much homologous antiserum during CIE to provide a clear picture. It may be that this strain, and the other two which did not produce detectable antigenic responses, were not highly immunogenic within the rabbits.

5.3.2. Studies on the cell wall proteins of C. difficile

Attention has been focussed on the cell proteins of C. difficile as a result of some of the work being done to develop methods for characterizing (or typing) different strains of the organism. The immunochemical finger-printing method of Poxton et al. (1984) and the radiolabelling employed by Tabaqchali et al. (1984) both rely on the presence or absence of particular protein bands in order to compare isolates.

Heard et al. (1986b) used immunoblotting to analyse further the nine strains of C. difficile previously characterized by ^{35}S-methionine radiolabelling (Tabaqchali et al., 1984). Antisera were raised against each of these nine strains which were then used to probe both the homologous strain and each of the other eight heterologous strains. It was found that there were three protein bands common to all of the strains when the serum

was tested against whole cell extracts of each strain. From the photographs in the paper I have calculated these to have M_rs of about 70kDa (CB_1), 58kDa (CB_2) and 55kDa (CB_3 - see Appendix 4 for standard curve used). These bands will be equivalent to the common antigenic bands of high M_r detected during our epidemiological investigations (Chapter 4). It was also reported that when a specific antiserum was immunoblotted against heterologous strains, the common bands were demonstrated but the bands specific to the heterologous strains were not.

From the studies done here I have demonstrated that each of the five strains studied (shown to be different by SDS-PAGE and immunoblotting of EDTA extracts) had between one to three major proteins of different M_rs extracted. I did think that these might correspond to one or other of the patterns of radiolabelling described by Tabaqchali et al. (1984). However on comparison of the M_r values it appears that only MPRL 1123 and MPRL 1128 had protein bands that were detected by this autoradiography technique. MPRL 1123 had a major band at 33.5kDa as well as two fairly prominant bands calculated to have M_rs of about 51.5 and 50kDa. From the figures in the Heard et al. (1986b) paper I have calculated that the two bands detected in strain W have M_rs of 51 and 33kDa. This might therefore be the same strain. Similarly, MPRL 1128 has a major protein band at 37kDa and another prominent band at

50kDa. Strain E, as defined by Tabaqchali et al. (1984), has two autoradiograph bands which have M_rs of about 49.5 and 37kDa. The major protein bands isolated from NCTC 11223, MPRL 604 and MPRL 683 did not correspond to any of the autoradiograph patterns described by this group. It may be that this particular amino acid is not present in these proteins or that it is present at only very low levels. Equally, these proteins may not have been synthesized over the period of labelling.

Minor proteins present in strains that had the same M_r values to the major proteins of other strains could have been assumed to be the same antigens. However few, if any, cross-reactions were demonstrated between these proteins. This observation further illustrates that although immunoblotting with antiserum raised against one strain of C. difficile can give a good indication of whether strains are the same or different, it will not fully characterize any strain unless it is entirely homologous (see section 4.3.1). This is a point also noted by Heard et al. (1986b). Immunoblotting with one antiserum cannot be used to characterize completely and 'type' an isolate.

Kawata et al. (1984) reported the presence of a regular array in the OWL of C. difficile strains. This group found that this regular array was composed of two main proteins which could have M_rs of 45 to 47kDa and 32kDa (9 strains) or 42kDa and 38kDa (2 strains). Again, only one

of the strains from which a urea extract was made (NCTC 11223) had major proteins that corresponded to these M_rs (46kDa and 32kDa).

The proteins extracted from NCTC 11223 were all very acidic in nature. My initial aim in applying the urea extract from this strain to a Chromatofocussing column was to try and separate the three proteins with M_rs of between 32kDa and 28.3kDa. Since they all focussed very closely together after SDS-PAGE I had hoped they might have sufficiently different pIs to allow separation on the basis of this parameter. However the results indicate that separation of the molecules was not very successful. The pH gradient of the column appears to have been correctly formed as the graph of fraction collected against pH of that fraction forms a straight line. I have subsequently learned that it is difficult to separate proteins of low pI by this system. It would perhaps have been better to try and separate the proteins by isoelectric focussing. Unfortunately there were technical problems with this technique that prevented the acquisition of any useful results.

Results of the hydrophobicity assays done here indicate that four of the five strains I was working with were relatively hydrophilic in nature. Of the 12 C. difficile isolates tested, only two (MPRL 604 and MPRL 678) appeared to be hydrophobic in nature. The fact that these two isolates, giving similar results, were shown to be

the same strain by immunoblotting was a useful control on the technique. Also, the S. aureus strain included gave a decrease in absorbance of about 85% which is what has been recorded for this organism in previous studies (Rosenberg et al., 1980).

Hydrophobicity is now generally considered to be a preliminary step towards bacterial adhesion. Wood-Helie et al. (1986) have previously reported that nine C. difficile strains they examined were hydrophobic in nature. This characteristic has been found to be common to several enteric pathogens and it has been suggested that it is an important prerequisite for colonization (Wadstrom et al., 1980). It is postulated that this characteristic may allow constant access of bacterial cells to the mucosa (overcoming electrostatic repulsion) while also serving as a counterforce to peristalsis. This group also suggested that the nonspecific attraction resulting from hydrophobicity was probably followed by a more specific adherence, both processes probably contributing to the colonization of the bowel. It may also provide a secondary reinforcement which counteracts the competition of free mucus for the receptors on epithelial cells (Freter, 1981).

Experiments employing hydrophobic interaction chromatography have shown that surface proteins of several pathogenic organims have a large number of nonpolar amino acids which are responsible for their

hydrophobic properties. Mutants lacking these proteins did not bind to the gel surface. Included in this group of organisms are E.coli which has colonization factor antigens I, II and 111, K88 and K99, Salmonella species, Campylobacter species and Yersinia enterocolitica (Wadstrom et al., 1980).

The hydrophobicity values obtained for cells will very much be influenced by the environment in which the cells are cultured. The availability of nutrients is likely to influence the character of the outermost cell envelope. In the study by Wood-Helie et al. (1986) it was found that all the species of clostridia examined grown on casamino acid (CAS) medium, showed some degree of adherence to polystyrene, but when grown on CAS containing clindamycin some species (i.e. C. perfringens, C. bifermentans) subsequently failed to adhere.

Given that the cell wall proteins of NCTC 11223 were highly charged when analysed by Chromatofocussing it might be expected that the organism would appear hydrophilic in nature. I did think that perhaps the original culture of the organisms in PPY had influenced the composition of the cell wall proteins since in the study by Wood-Helie et al. (1986) organisms were grown on solid media on which the environment may have been much less acidic. I found the pH drop in an overnight PPY culture to be from about pH 7.0 to pH 5.9. This was the same for all cultures and would therefore not explain the

difference in the hydrophobicity values obtained for MPRL 604 and MPRL 678. However, it was interesting to see that culture of MPRL 604 on BA did decrease its apparent hydrophobicity value by about a third.

The greater hydrophobicity detected with MPRL 604 and MPRL 678 cannot be related directly to the virulence of these organisms. One organism was isolated from a patient with mild diarrhoea, the other from another with severe symptoms. As discussed in section 5.1, many factors can contribute to the disease symptoms occurring in individuals; it is unrealistic to label any one surface component or physiological aspect of a cell and say this is the only factor involved in the production of symptoms. Pathogenesis is now well known to be a multifactorial process involving a variety of different virulence factors (Smith, 1978). It may also be the case that the immune response in these patients was different, so influencing the severity of symptoms .

5.3.3. The flagella of C. difficile

The motility and chemotactic ability of flagella has been shown to be associated with the virulence of Gram-negative aerobes such as V. cholerae (Guentzel and Berry, 1975; Yancy et al., 1978) and P. aeruginosa (Craven and Montie, 1981; Montie et al., 1982). The flagella of these bacteria are known to play an important role in inducing host protection against infection (Yancy

et al., 1979; Holder et al., 1982). The role of flagella in the virulence of anaerobic organisms does not appear to have been studied very extensively. However studies on one strain of Clostridium have been reported. Investigations on the role of the flagella of C. chauvoei in raising protective antibodies in mice have produced conflicting results. Chandler and Gulasekharam (1974) stated that the highly protective antigen of this organism was not flagellar in origin. In contrast Stevenson and Stronger (1979) reported that the flagella did appear to induce a highly protective antibody response.

The method used here for isolation of flagella from C. difficile resulted in concentration of the appendages in one or two closely associated bands after caesium chloride density gradient centrifugation. Chandler and Gulasekharam (1974) reported that one such dense band was seen after this treatment of C. chauvoei. When the two fractions collected from each strain were run on SDS-PAGE it was apparent that each fraction contained protein of the same M_r. When viewed by EM, following shadow casting, the only material seen in each of the fractions was the flagella. Immunoblotting of each extract against its homologous antiserum showed that the appendages produced an antigenic response within the rabbits. Antigenic cross reaction was also demonstrated between the flagella preparations from the two strains. A very weak response

was also detected with antisera against another four strains of C. difficile. No antigenic response was seen when the protein was probed with antiserum against NCTC 11223. It is very probable that the flagella of this strain do not elicit a very strong antigenic response. This strain of the organism is only sluggishly motile and there was only a very faint band apparent after the caesium chloride centrifugation.

The chemical composition of the flagella in strictly anaerobic bacteria has been little studied. However in facultatively anaerobic or aerobic bacteria, with peritrichous or monotrichous flagella, the filament protein (flagellin) is reported to be a multimer of a single polypeptide with antigenicity and amino acid composition differing from the proteins of the other structural components (Dimmit and Simon, 1970; Kagawa et al., 1976). From the results obtained here I would suggest that the flagella extracted from both of the C. difficile strains were composed of one single protein, as demonstrated by their antigenic cross-reactivity. The observed division of this protein within the caesium chloride gradient (which was reproducible) may have arisen as a result of the flagella forming aggregates. However if such aggregates did exist they were not separated during SDS-PAGE even after extensive boiling. The precise nature of these C. difficile flagella remains uncertain.

Concluding remarks

The occurrence of both common and unique antigens within strains of C. difficile has been demonstrated. The membrane carbohydrate antigen is of a novel nature, different from that isolated from other Gram-positive organisms such as staphylococci, streptococci, lactobacilli and Bacillus species (Wicken and Knox, 1975). It has been shown that there is a variety of proteins present in the cell walls of the organism. Indeed, there is possibly a greater variety than has been previously reported. It also appears that motile strains of the organism contain flagella of a common nature as demonstrated by their antigenic cross-reactivity. Further work to elucidate the precise role and structure of proteins in the cell walls and to determine whether any of the antigens detected in these studies are important in protecting the host from infection, should be done in the future.

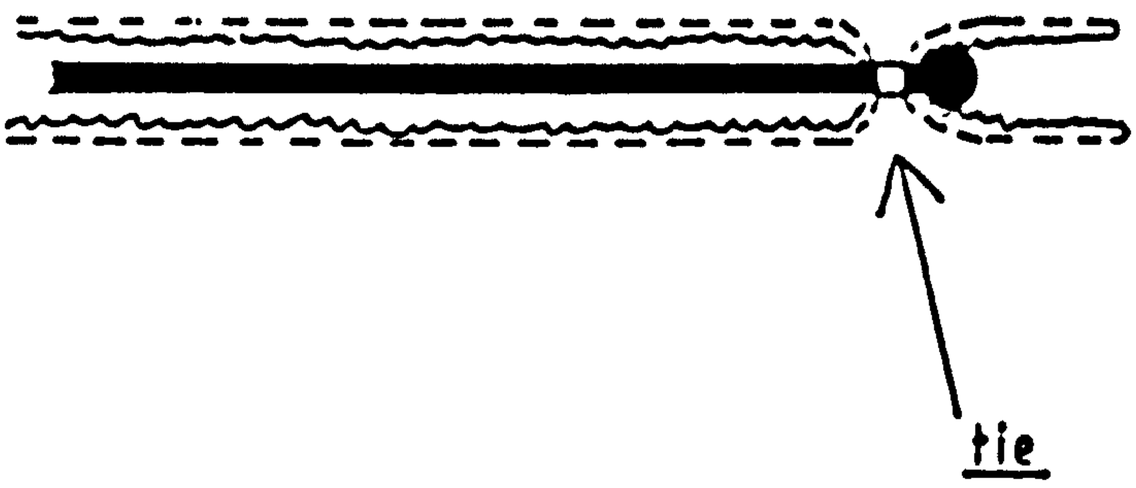
5.4. Adhesion studies

During all these studies broths, diluents and agar plates were pre-reduced before use on the bench.

5.4.1a Investigations into the association of C. difficile with gut mucosa

The ileum was removed from a C3Hb mouse (or two, if required) and was pushed onto a glass rod and tied at one end. The gut was subsequently everted over the rod as shown in Fig. 5.13, the contents being collected into 2ml of Krebs-Ringer (KR) solution (see Appendix 1). The everted ileum was rinsed with tissue culture medium (TCM - RPMI 1640 - Gibco, Paisley, Renfrewshire), pH 7.3, containing 20mM Hepes buffer; the pH of this was adjusted with 1M HCl after addition of the Hepes. The gut was cut into six pieces each 4.5cm in length. Studies showed that this length of freshly excised and everted gut took up between 0.0163ml and 0.0209ml of fluid (six pieces of gut measured) with a mean value of 0.0192 (0.0015 standard deviation).

Five of these pieces of gut were secured onto glass rods, with threads tied a distance of 4cm apart. Each was retained in TCM. The sixth piece was ground up with a tissue grinder in 2ml of KR solution; 0.1ml of this was spread onto a CCFA plate to provide a time zero negative control. A similar volume of the gut contents solution was spread onto another CCFA as a further control. The



mucosal surface of ileum

serosal surface of ileum

Fig. 5.13 Eversion of mouse ileum over a glass rod.

other five pieces of gut were individually introduced into separate test-tubes containing 10ml of a 3h culture of C. difficile MPRL 1121, adjusted to a concentration of about 1 x 10^7 organisms/ml in TCM (after a total count had been done). This culture was derived from a 5% inoculum of an overnight PPY culture into fresh p-r PPY. One piece of ileum was dipped into this suspension momentarily, only long enough to wet the entire surface of the tissue (T_o, about 2 sec). The other five pieces were left in the suspension, on a shaker, for either 5, 10, 15, 30 or 60 minutes. After removal, each piece was given four successive 10 sec washes, with gentle agitation, in 25ml of KR solution (in Universal bottles) and was finally cut from the glass rod and ground up with 2ml of KR solution. A sample (0.1ml) of each wash solution and of the homogenized gut (plus any dilutions made in KR solution) was spread onto a CCFA plate. These were incubated for 48h before colony counts were done.

Tables 5.8a and 5.8b record the total number of organisms present in each of these four wash solutions and on the gut in two such experiments (A and B). These figures were calculated as shown below:

For the washes: a x dilution factor x 10 x 25

For the homogenized gut: a x dilution factor x 10 x 2

where a is the total number of colonies per plate.

TABLE 5.8a: Experiment A

Total number of viable C. difficile recovered from washes and ileal tissue with time

Time in Cd suspension	Number of viable organisms in Wash 1	Wash 2	Wash 3	Wash 4	Homogenised gut	Total organisms recovered	Percentage association
T_o	7.25×10^4	2.27×10^4	7.00×10^3	2.75×10^3	8.80×10^3	1.14×10^5	7.7%
5 min	1.48×10^5	3.35×10^4	1.33×10^4	5.50×10^3	5.12×10^4	2.52×10^5	20.2%
10 min	1.67×10^5	6.40×10^4	2.95×10^4	2.05×10^4	1.02×10^5	3.83×10^5	26.6%
15 min	2.53×10^5	7.40×10^4	4.60×10^4	3.53×10^4	1.58×10^5	5.66×10^5	27.9%
30 min	3.80×10^5	1.14×10^5	7.43×10^4	4.33×10^4	2.82×10^5	8.94×10^5	31.6%
60 min	5.18×10^5	9.68×10^4	8.25×10^4	5.10×10^4	3.68×10^5	1.11×10^6	33.1%
predicted number at T_o	1.65×10^5	3.17×10^3	61	1	zero	1.68×10^5	...

TABLE 5.8b: Experiment B

Total number of viable C. difficile recovered from washes and ileal tissue with time

Time in Cd suspension	Number of viable organisms in Wash 1	Wash 2	Wash 3	Wash 4	Homogenized gut	Total organisms recovered	Percentage association
T_o	1.55×10^5	1.18×10^4	1.05×10^4	5.00×10^3	1.16×10^4	1.94×10^5	5.9%
5 min	2.03×10^5	2.28×10^4	1.63×10^4	8.25×10^3	5.50×10^4	3.05×10^5	18.0%
10 min	2.35×10^5	3.25×10^4	1.88×10^4	1.25×10^4	8.80×10^4	3.87×10^5	22.7%
15 min	2.85×10^5	4.25×10^4	3.08×10^4	1.70×10^4	1.38×10^5	5.13×10^5	26.9%
30 min	4.50×10^5	1.00×10^5	3.58×10^4	1.83×10^4	2.92×10^5	8.96×10^5	32.6%
60 min	5.60×10^5	2.85×10^4	7.50×10^4	6.40×10^4	3.96×10^5	1.12×10^6	35.2%
predicted number at T_o	2.75×10^5	5.28×10^3	1.01×10^2	2	zero	2.80×10^5	...

The total number of colonies per plate in both experiments are noted in Appendix 3. The column marked percentage association (Pa) is derived from the following calculation:

$$Pa = \frac{\text{Viable organisms on homogenized gut}}{\text{Viable organisms in wash 1 + wash 2 + wash 3 + wash 4 + homogenized gut}}$$

This figure gives an indication as to the actual levels of association of C. difficile with the ileal mucosa, given the possible number of organisms that were in contact with the gut at the time it was removed from the C. difficile suspension. Tables 5.8a and 5.8b also indicate the number of cells that would be expected in each of the washes assuming that 0.0192ml of fluid was transferred to each piece of gut from the original C. difficile suspension and from one wash to the next.

Viable counts were done on the C. difficile suspension used during the course of Experiment B (two plates per count). There were originally 1.43×10^7 viable cells/ml at T_o but after 30 min this figure had fallen by 57.3% (to 6.10×10^6) and after 60 min it had fallen by 74.1% (to 3.7×10^6). The concentration of C. difficile at T_o in experiment A was 8.60×10^6 viable organisms/ml.

No C. difficile were recovered from either of the negative control plates. It is clear that the total

number of organisms being transferred from the C. difficile suspension on the pieces of gut (washes 1, 2, 3 and 4 plus the homogenized gut figure), increased the longer the tissue was left in contact with the organisms. After 60 min the number of organisms transferred was 9.7 times that recovered at T_o in experiment A and 5.8 times the T_o figure in experiment B. The majority of cells transferred were removed in the first wash (63.6% in experiment A and 79.9% in experiment B at T_o, 44.7% and 55.6% respectively after 15 min and 46.7 and 50% after 60 min). At each time interval the subsequent washes had decreasing numbers of cells in them (Fig. 5.14).

In both experiments the total number of organisms recovered during the processing of the gut at T_o was about 70% of the predicted carry over into the first wash. This may indicate that the pieces of gut which were kept moist prior to use were not in fact absorbing as much fluid as was predicted by the measurements with freshly excised tissue. It is also apparent that the subsequent washes, 2 to 4, contained many more organisms than predicted.

The number of organisms remaining associated with the gut after the washes increased with time (Fig. 5.15). There appears to be a linear increase in numbers over the first 30 min followed by a levelling off in the rate of increase by 60 min. The Pa value is also seen to increase with time and begin to level off after 30 min.

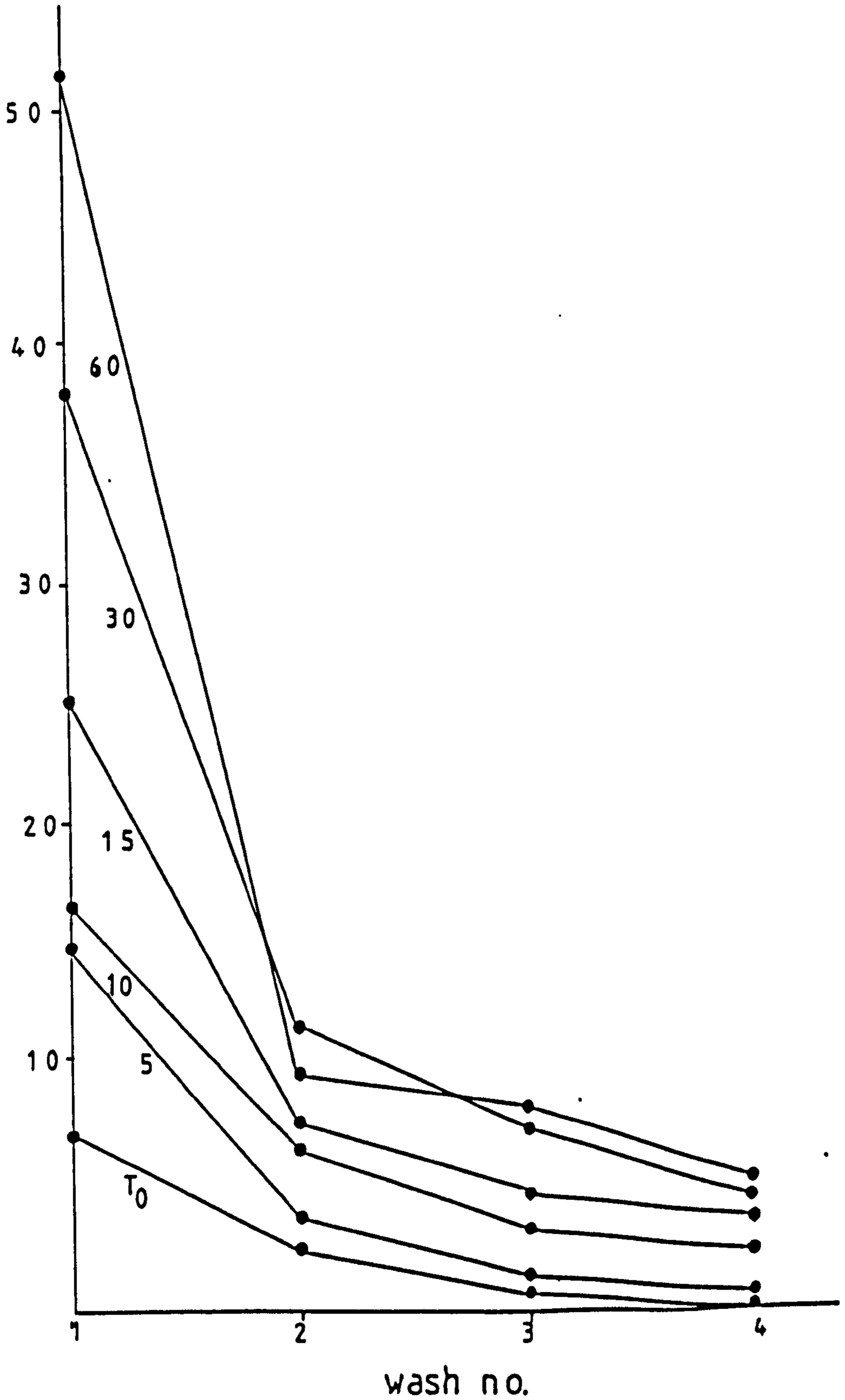


Fig. 5.14 Graph of viable organisms recovered from each wash after removal of ileal tissue from a C. difficile suspension at the time specified (T_o to 60 min). Figures are taken from Table 5.8a. A similar graph was obtained with the results in Table 5.8b.

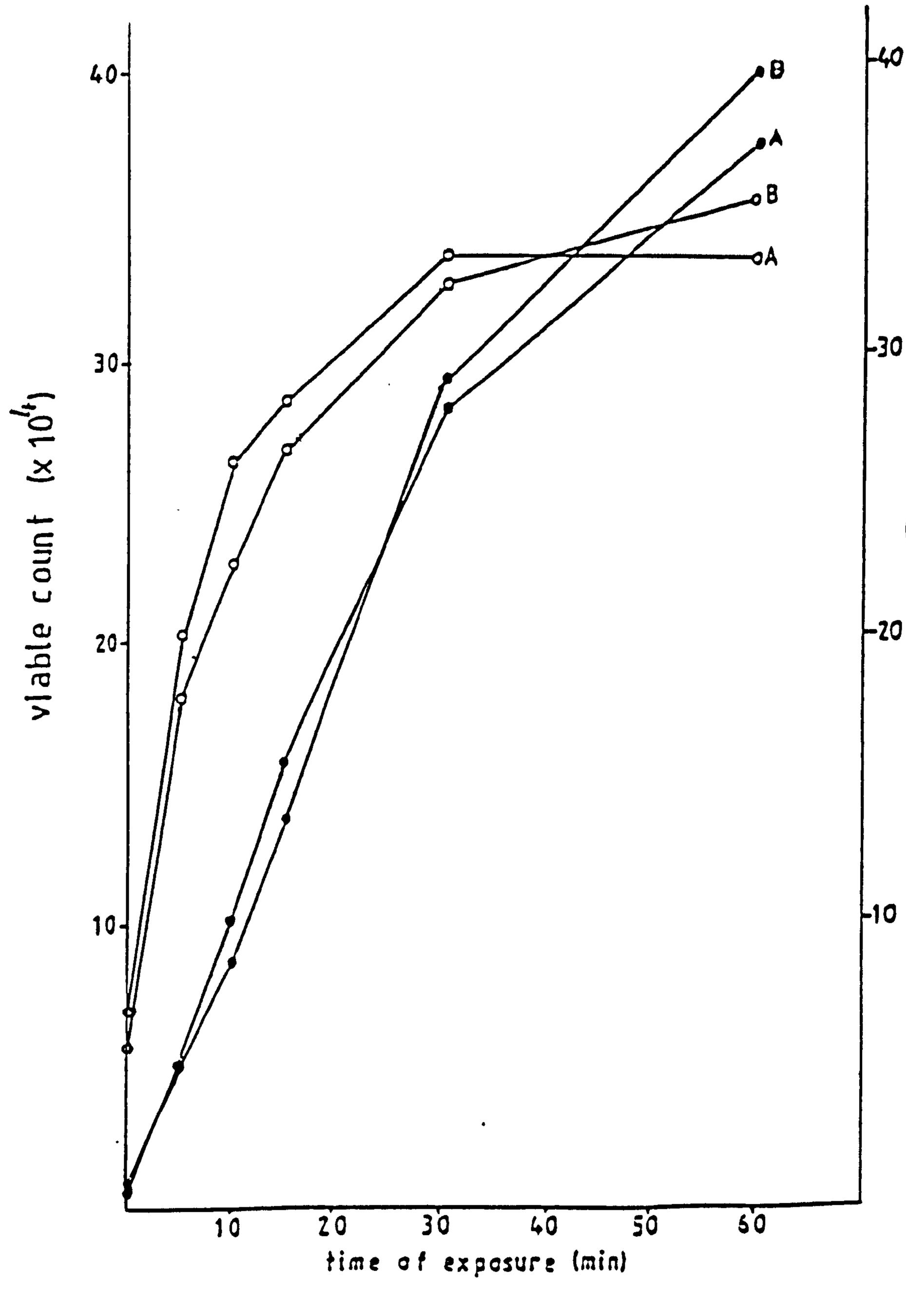


Fig. 5.15 Graph of viable organisms recovered with increasing exposure of mouse ileum to C. difficile. Also recorded is the Pa value for each time interval. Figures are derived from Tables 5.8a (A) and 5.8b (B).

Two questions arose from these results. Firstly, was this pattern of association of C. difficile with the gut tissue unique to this organism and secondly, was the association a competitive event? To try and answer these questions the experimental procedure was repeated with a Bacteroides fragilis strain. The results of this experiment are described below.

5.4.1b Association of B. fragilis with gut mucosa; a comparison with C. difficile

The B. fragilis strain used in this work (MPRL 1282) was originally chosen as it had an unusual antibiotic resistance profile which would have made detection on selective agar easy. However, as it turned out the organism was quite easily identified by colony appearance on BA, as no organisms cultured from the ileum looked similar to it.

The adhesion experiment was repeated exactly as described previously for the C. difficile and the results obtained are shown in Table 5.9. Viable counts done on the B. fragilis suspension used indicated that at T_o there were 5.65×10^6 organisms/ml and that there was no significant change in this count during the experiment. The predicted recovery of cells from 0.0192ml of this suspension are also included in Table 5.9.

The total number of cells transferred on the gut surface increased with time but this increase (2.7 fold in the 60

TABLE 5.9

Total number of viable B. fragilis recovered from washes and ileal tissue with time

Time in Bf suspension	Number of viable organisms in Wash 1	Wash 2	Wash 3	Wash 4	Homogenized gut	Total organisms recovered	Percentage association
T_o	3.10×10^5	1.10×10^4	3.25×10^3	3.00×10^3	3.20×10^3	3.30×10^5	0.9%
5 min	3.80×10^5	2.10×10^4	7.00×10^3	6.25×10^3	2.80×10^4	4.42×10^5	6.3%
10 min	4.35×10^5	3.68×10^4	1.35×10^4	1.28×10^4	5.20×10^4	5.50×10^5	9.5%
15 min	4.58×10^5	1.55×10^5	1.45×10^4	1.10×10^4	9.20×10^4	7.31×10^5	12.6%
30 min	4.93×10^5	9.65×10^4	6.50×10^4	5.80×10^4	1.16×10^5	8.29×10^5	14.0%
60 min	5.30×10^5	1.20×10^5	5.80×10^4	4.98×10^4	1.28×10^5	8.86×10^5	14.4%
predicted number at T_o	1.08×10^5	2.07×10^3	39	zero	zero	1.10×10^5	...

min) was not as great as that observed with the C. difficile. The actual number of organisms remaining associated with the ileum after washing also increased giving a similar curve to that of C. difficile (Fig. 5.16). However, the number of organisms recovered started to level out after 15 rather than 30 min. The same effect was found with the Pa values. These Pa values are all lower than those calculated for C. difficile at the corresponding times.

The number of B. fragilis recovered at any particular time from the homogenized gut was always less than the corresponding C. difficile figures, ranging from about 30 to 65%. However this was probably a consequence of the B. fragilis viable count being only 38% (experiment B) to 66% (experiment A) of the C. difficile viable counts.

The B. fragilis cells available in suspension were much more rapidly taken up by the surface of the gut at both T_o and after 15 min - T_{15} (Table 5.10). However the percentage increase in the actual number of organisms recovered from the homogenized gut over time was of the same order of magnitude as with the C. difficile (Table 5.11).

Figure 5.17 shows the pattern of recovery of B. fragilis from the washes was similar to that of C. difficile. There was a greater percentage of the total cells transferred lost in the first wash at T_o (93.9% compared to 63.6% and 79.9% for C. difficile). By 15 min the

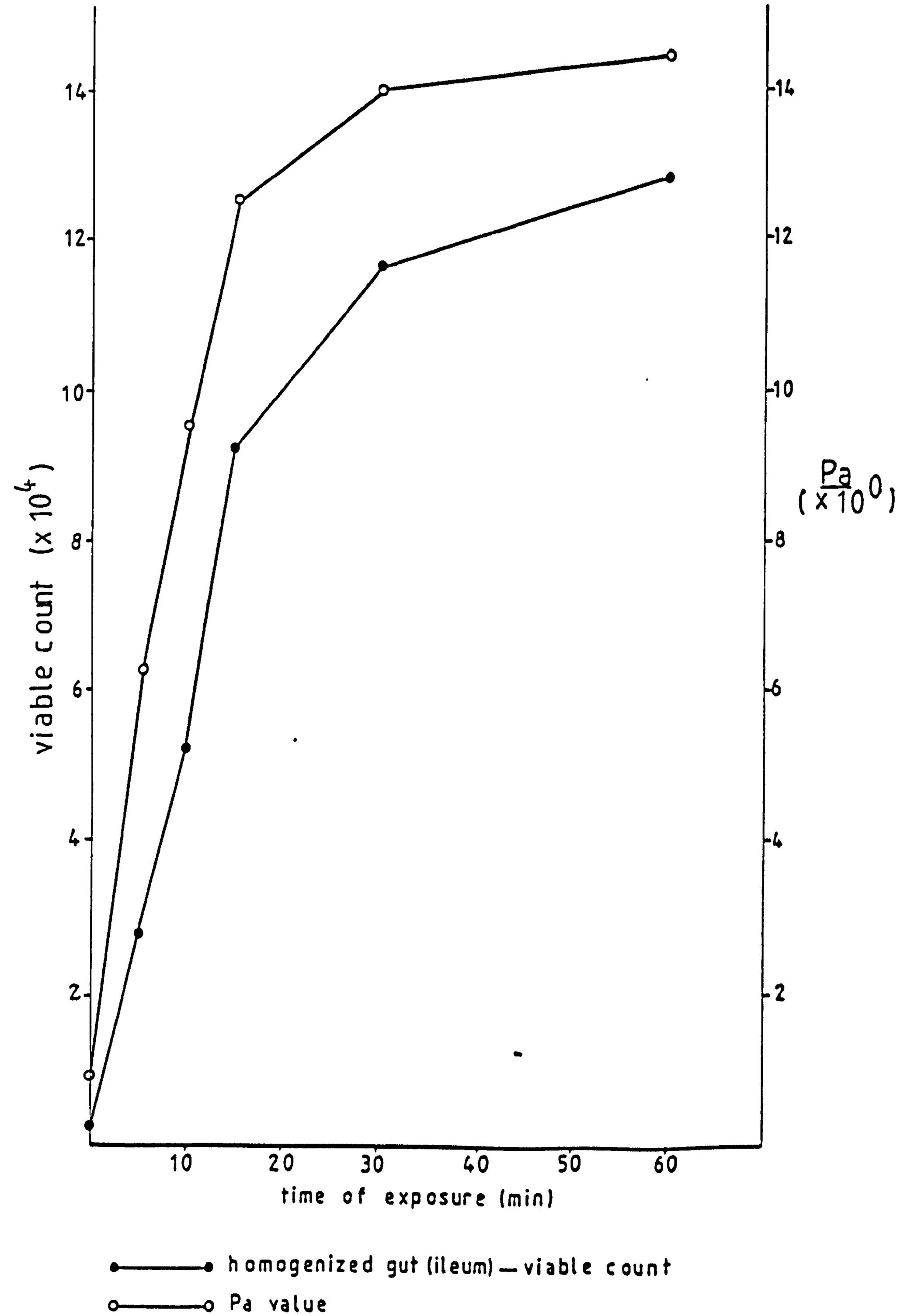


Fig. 5.16 Graph of viable organisms recovered with increasing exposure of mouse ileum to B. fragilis. Also recorded is the Pa value for each time interval. Figures are taken from Table 5.9.

TABLE 5.10

Comparison of the total C. difficile and B. fragilis recovered as a percentage of the viable count over the first 15 minutes of adhesion experiments

Organism	Experiment & Viable count	Time	Total cells recovered / Viable count	Pa*
C. difficile	Experiment A 8.60×10^6	T_o	1.32%	7.7%
"	"	T_{15}	6.58%	27.9%
C. difficile	Experiment B 1.43×10^6	T_o	1.36%	5.9%
"	"	T_{15}	3.59%	26.9%
B. fragilis	Table 5.9 5.65×10^6	T_o	5.8%	0.9%
"	"	T_{15}	12.9%	12.6%

*: Pa (percentage association) values are included for comparison

TABLE 5.11

percentage increase in the number of organisms recovered from homogenized gut with time

Time interval	percentage increase in recovery of organisms*		
	B. fragilis	C. difficile	
		Experiment A	Experiment B
T_o - 15 min	2775%	1695%	1090%
T_o - 30 min	3525%	3105%	2417%
T_o - 60 min	3900%	4082%	3314%

*: calculated as $\frac{T_x - T_o}{T_o} \times 100$

where T_x is the homogenized gut count after 15, 30 or 60 min.

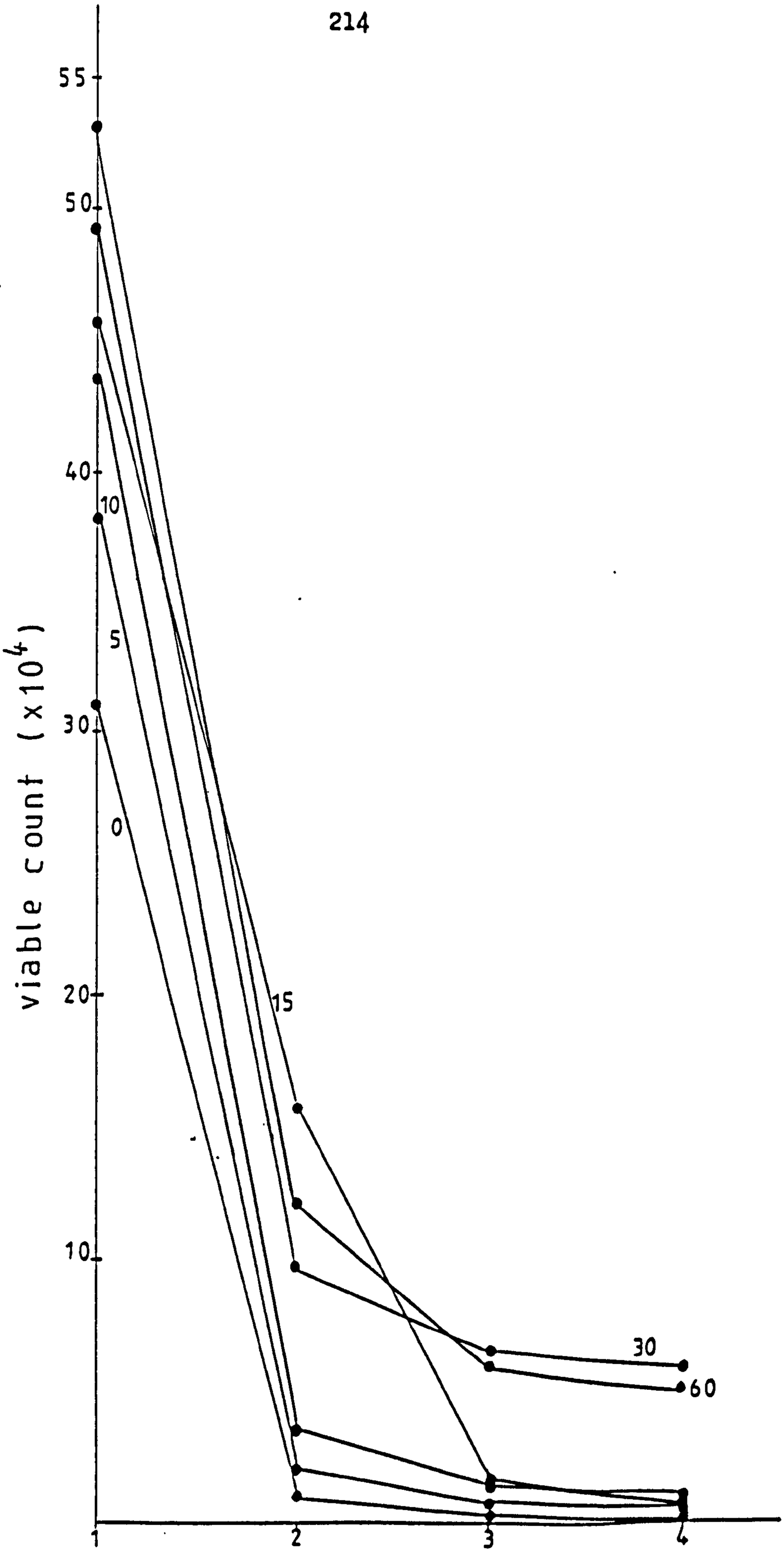


Fig.5.17 - see over for legend.

Fig. 5.17 Graph of viable organisms recovered from each wash after removal of ileal tissue from a B. fragilis suspension at the time specified (T_o to 60 min). Figures are taken from Table 5.9.

percentage recovered in this first wash levelled out at around 60%.

A further experiment was done to see whether prior treatment of a piece of gut in a B. fragilis suspension would result in a decrease in the association of C. difficile with the ileal tissue. To try and get maximum association of B. fragilis with the tissue the organism was used at a higher concentration than the C. difficile since a) it was much smaller than the C. difficile cells and b) in the previous experiment fewer B. fragilis were actually recovered from the gut than C. difficile.

Ileal tissue was placed in a suspension of B. fragilis (viable count of 5.00×10^8 organisms/ml) for 15 min and then transferred, with no washing, to a suspension of C. difficile at a concentration of 8.85×10^6 organisms/ml for a further 15 min. The number of B. fragilis and C. difficile recovered from the gut after this are recorded in Table 5.12. Results of control experiments, measuring recovery of each organism from individual pieces of gut at T_o and T_{15}, are also included.

Taking the control experiments first it can be seen that the C. difficile behaved in the same manner as observed previously. The number of cells lost in the first wash at T_o was 70.8% of the total recovered at this time and the actual increase in recovery of organisms from the gut over the 15 min was 1508%. The total number of organisms

TABLE 5.12

Number of viable organisms detected in experiment to assess the effect of prior treatment of ileum with B. fragilis on the association of C. difficile with ileal tissue

Organism	Time	Number of viable organisms in[a] Wash 1	Wash 2	Wash 3	Wash 4	Homogenized gut	Total[a] organisms recovered	Pa
Combined experiment								
B. fragilis	T_{15}[b] recovery	833	43.8	33.0	27.5	11.2	949	1.18%
C. difficile	T_{15}[b] recovery	22.8	3.03	2.03	0.875	7.70	36.4	21.2%
Control experiments								
B. fragilis	T_o	475	24.5	4.93	2.95	6.02	513	1.17%
B. fragilis	T_{15}	900	82.5	34.0	22.0	16.0	1050	1.52%
C. difficile	T_o	8.85	1.03	0.725	1.00	0.920	12.5	7.36%
C. difficile	T_{15}	30.0	8.65	4.83	2.33	14.8	60.6	24.4%

a: all viable count figures require to be multiplied x 10^4

b: recovery of organisms from gut following exposure to B. fragilis for 15 min followed by C. difficile for 15 min.

transferred on the pieces of gut increased by 4.8 fold. The B. fragilis suspension behaved differently from the previous experiment with this organism where 5.65 x 10^6 organisms/ml were used. Now, with the cells at 88 times this concentration, the actual increase in recovery of the organisms from the gut over the 15 min was only 166% compared with 2775% previously. As many more organisms were recovered at both T_o and after 15 min in this experiment this could indicate that more of the binding sites were taken up by the B. fragilis at T_o and that the potential for further uptake was reduced.

The results of the combined experiment show that the presence of the B. fragilis on the gut has, compared to the control T_{15} experiment, reduced both the total number of C. difficile transferred (by 40%) and the number of organisms finally recovered from the gut (by 48%).

In order to assess whether or not C. difficile would associate with cells lacking mucus, attempts were made to remove this layer from the everted gut. Attempts to scrape the mucus off by purely physical means with a scalpel were not successful. Subsequently, chemical removal of the layer was attempted. This involved the addition of the gut to 25mg/ml of N-acetyl-L-cysteine (Sigma) in 0.5M phosphate buffer, pH 7.0. This is a mucolytic agent which was reported to be very effective at removal of mucus from tissue (Professor G. Cooper,

University of New South Wales, Australia - personal communication). The pieces of gut were placed in this solution on a magnetic stirrer for either 30 or 60 min at 37°C. Following this the gut was collected into formalinized saline prior to preparation of paraffin-embedded transverse tissue sections (see sections 2.34 and 2.35). When the stained sections were viewed with the light microscope it was apparent that although much of the mucus had been removed during this treatment there was still a relatively large proportion remaining in association with the cells. Results obtained from experiments with this treated gut were inconsistent and showed no clear pattern, most probably as a result of uneven distribution of mucus on the pieces of gut.

5.5. Discussion

Adhesion has been shown to increase the virulence of Gram-negative toxigenic organisms such as *V. cholerae* (Schrank and Verway, 1976) and *E. coli* (Jones and Rutter, 1972). In this work I wanted to assess the potential role of adhesion in the pathogenesis of *C. difficile*-associated disease since this is also a toxigenic organism.

General confusion exists regarding whether or not *C. difficile* adheres to the gut mucosa. Histological examination of colonic biopsy material from cases of PMC does not, in the vast majority of reports, show the presence of bacterial cells within the characteristic plaques. There is however one report (Borriello, 1979) which does suggest that the organisms might adhere to colonic tissue.

Barer (1984) reported apparent adhesion of *C. difficile* to intestinal 407 cell monolayers. I would tend to question the relevance of this study relying as it did, upon the use of a cell line. It is well appreciated that such cells have altered surface characteristics and they are known to have atypical adherence properties. Consequently, I would suggest that any results obtained with such cells should be interpreted with caution. To avoid these potential problems I wished to use an assay system that would determine association of *C. difficile*

with normal gut epithelium.

Ileal tissue was used for these studies as it was easy to obtain and use rapidly. Colonic material would have required considerable washing and would probably have still been covered with many commensal organisms. Comparatively few organisms were associated with the ileal mucosa and those present could not be confused with the C. difficile growing on CCFA and B. fragilis cultured on BA. Finally, since the toxins produced by C. difficile affect ileal as well as colonic cells (Mitchell et al., 1986) it seemed reasonable to use this tissue for the studies. The C. difficile strain used (MPRL 1121) was chosen as it was known to be pathogenic in animals (Dr. S.P. Borriello - personal communication).

The results show that more viable cells from the C. difficile suspension were removed on the surface of the ileum the longer the tissue was left in contact with the bacteria. Barer (1984) reported that it took at least 90 min before there was association of C. difficile with cell monolayers. In comparison, the results here indicate that there was very rapid association. This may well have been facilitated by gut mucus which would not have been present on the cell line.

In both the C. difficile experiments (A and B) the figures for total recovery are very similar at each of the time intervals. Fewer cells than expected were recovered in wash 1 at T_o but, as mentioned previously,

this may have been due to less of the bacterial suspension actually being taken up by the tissue than was predicted. About 60 to 70% of the total cells recovered at T_o were in wash 1; by 15 min this figure had fallen to around 45 or 55% and was about 50% after 60 min. This could indicate that the cells actually transferred on the gut surface were sticking more thoroughly following longer periods in the bacterial suspension.

The number of organisms remaining associated with the gut after washing increased with time as did the Pa values. The levelling off seen in these figures may have arisen as a result of the ileal tissue shedding mucus and/or cells during the washing steps. Pieces of the mucosa could be seen, evenly dispersed throughout the wash solutions, after 30 and 60 min and it is possible that such loss of material was occurring at the earlier stages in the experiments. It is certainly clear that many more organisms were recovered in washes 2 to 4 than were predicated at T_o. It may therefore be that the figures obtained for organisms remaining associated with the homogenized gut are artificially low as a result of pieces of the mucosa being shed during the wash process. The decreasing rate of association may also indicate that all the potential binding sites on the gut had been utilized. An experiment with greater initial numbers of cells would have shown whether this was the case if the levelling off in association occurred more rapidly (see

later discussion on B. fragilis).

This levelling off in association of C. difficile with the gut may also have been a result of the decreasing viability of the cells. This being the case, the observed increases in association are all the more significant. It would have been preferable to do these experiments within an anaerobic cabinet but a sufficiently large one was not available.

The association of the B. fragilis with the gut tissue showed some differences. To begin with, a greater percentage of these cells were taken up on the surface of the gut relative to the initial number of cells present in suspension. However, compared to the C. difficile a greater proportion of these cells were removed in the first wash over the entire 60 min. This would indicate that the cells actually picked up on the surface of the gut were more easily removed than the C. difficile. The lower Pa values calculated also suggest that of all the cells transferred on the surface of the tissue, fewer actually remained in association with it after washing. It therefore appears that although more B. fragilis can loosly associate with the gut, this organism does not have the ability to 'stick' to the surface as thoroughly as C. difficile.

The levelling off in Pa value and number of cells recovered from the gut after 15 min cannot be attributed

to decreasing viability of the B. fragilis culture. In this experiment it is more likely to be due to a saturation of available binding sites. This argument is borne out by the fact that only 1.7 times as many B. fragilis cells were recovered from the washed gut in the later experiment employing 5.00×10^8 organisms/ml compared to this one with only 5.65×10^6 organisms/ml.

The final experiment done indicated that prior treatment of the ileum with a high concentration of B. fragilis cells could result in a decrease in subsequent recovery of C. difficile from the gut. The fact that the Pa value in the combined experiment was similar to the T_{15} control Pa value indicates that those cells that did manage to associate did so in the same manner as in the control experiment. Since a greater proportion of cells were not recovered in the washes this would suggest that the C. difficile cells did not even loosly associate with those sites on the tissue previously taken up by the B. fragilis. It may therefore be that although C. difficile does have the ability to associate with the gut mucosa, it cannot actually do this when the gut surface is already covered with other organisms as in the situation in vivo. It would have been interesting to see if antibiotic treatment of organisms already associating with the gut mucosa would have increased the eventual recovery of C. difficile.

It is probable that the association observed between the

gut and C. difficile or B. fragilis was facilitated by the mucus layer, at least in the initial stages of association. It would have been useful to compare the results obtained with similar experiments done with non-mucus covered cells. However it may be that the only way to do this would be to use a cell line which, as mentioned at the beginning of this discussion, may not be worthwhile. Alternatively, chemical removal of the mucus might be more successful if the gut was kept in oxygenated conditions and left in an N-acetyl-L-cysteine solution for longer before transferring it to anaerobic conditions for the actual experiment. The use of a fluorescent label against a C. difficile surface antigen could be helpful in detecting precisely where the organism associates with the tissue.

All the experiments done here were with the same strain of C. difficile; it would be interesting to investigate other strains for comparison. Further investigations into the effect of normal gut commensals on association, perhaps by use of mixed bacterial suspensions, might provide greater insight into the potential for association of C. difficile with the gut mucosa in vivo.

CHAPTER 6

FINAL COMMENTS

"It is a riddle wrapped in a mystery inside an enigma"

Sir Winston S. Churchill

Many unanswered questions remain regarding C. difficile and its role in the pathogenesis of bowel disease. When initially isolated from patients with AA-PMC, it seemed that it would be a simple task to correlate the occurrence of this organism with the disease and that the major virulence factor would be the easily detectable cytotoxin. Clearly, the increasing research done since then, has shown that things are far more complicated than this. The organism has been found associated with several other less severe bowel disorders and other factors have emerged as potentially important in virulence.

The investigations undertaken in this thesis fell into three main areas:

1) studies on cultural methods for the organism
2) epidemiological investigations
3) isolation and immunochemical investigation of surface components.

Most of the cultural investigations undertaken centred around attempts to isolate C. difficile from as high a percentage of healthy individuals as possible. It is apparent that the organism may be present at exceedingly low levels in the healthy gut, making isolation without

extensive enrichment improbable.

Based on these results we are no nearer providing a definitive answer as to whether or not C. difficile is a potential endogenous pathogen in all individuals. We can say that it does exist at very low levels in a higher percentage of individuals than previously reported in the majority of investigations. It may be the case that only certain people are colonized by the organism but the factors which might determine this are not readily apparent. Given appropriate conditions these organisms may give rise to a pathogenic effect. In this study tetracyclines and penicillins were not found to be among these factors.

Enrichment culture is not routinely used here in the diagnostic laboratories, for purely quantitative reasons. If the organism is associated with symptoms it should be present at high enough concentrations for direct detection by plate culture. Occasional specimens used to be enriched (where C. difficile infection was suspected although the organism was not cultured on CCFA). However now that use of 1/2 CCFA gives greater isolation rates and as a result of my enrichment studies on normal individuals, this is no longer considered worthwhile. Any organisms recovered by enrichment in such a situation could have been present at low levels before the patient developed GI symptoms.

Results of the epidemiological studies were again inconclusive, although nontheless interesting. The MRU investigation was the largest and provided a useful study of the organism within an immunocompromised group. In this instance it did look as if the broad spectrum oral cephalosporins used in the treatment of many of the infections in these patients may well have been implicated in facilitating disease.

It would be interesting to study both patients and isolates recovered from individuals with cystic fibrosis. This would allow the study of organisms isolated in the presence of antimicrobial agents that are not associated with symptoms. It may be in such patients that the altered physiological condition of the gut influences the outcome of infection.

In conclusion it must be accepted from the evidence currently available that disease can arise either as a result of cross infection or be an endogenous entity. The outcome will in all probability be dependent on a number of other environmental and physiological factors.

The main work in this thesis concerned studying the bacterial cell surface, assessing its potential antigenicity and role in virulence. As discussed in Chapter 5 no 'virulence factor' can be looked at in isolation. For any organism to be pathogenic it requires to have a variety of different factors that will permit

it to a) invade b) associate c) elicit damage and d) resist any immune response. Consequently, many areas of investigation are required in attempting to define what makes a particular organism pathogenic.

Interesting points have been raised from the work done here. There are clearly many potential antigens that should be investigated further to assess their precise functions. Is, for example, the novel LTA-type molecule described involved in pathogenicity either as a direct adhesin (as in streptococci) or by providing a hydrophobic molecule within the cell surface? The flagella could well be important in virulence as already illustrated with other pathogenic organisms. Studies are currently underway in this laboratory to assess whether all motile strains of C. difficile might possess the same flagellar antigen. It would be interesting to know if different C. difficile strains all possess a regular array and if so whether or not the variety of proteins seen in the extracts prepared here is reflected in the composition of this layer.

Work done on the toxins produced by C. difficile has gained increasing importance over the period of this project. Of all these molecules the enterotoxin (toxin A) is emerging as potentially the factor most likely to elicit the gross physical effects encountered in C. difficile-associated pathogenicity.

I would like to conclude with a short summary of three

papers recently published by Mitchell et al. (1987a, 1987b and 1987c) describing work this group have done to try and elucidate the action of this toxin (and also toxin B) on the gut mucosa.

6.1. An update on the possible mode(s) of action of C. difficile toxin(s) on ileal and colonic tissue

It seems that the damage induced by toxin A is histological rather than biochemical in nature. A decrease in protein synthesis was observed in both rabbit ileum and colon but this was associated with a loss of protein synthesizing villus cells from the mucosa rather than decreased protein synthetic ability. Also there was no effect on membrane integrity/permeability in isolated intestinal cells treated with the toxin.

The kinetics of the resulting tissue damage were very different in the two tissues. A much longer incubation period (4h) with toxin A was required for fluid accumulation to occur in the colon, compared with 45 min in the ileum. Fluid secretion into the gut lumen was induced only when toxin had gained access to deeper tissues. It is speculated that this may be achieved by several cycles of toxin uptake, with greater tissue damage occurring after each one. Only after a given amount of time would the accumulated damage finally result in fluid secretion. Toxin A induced haemorrhage in both ileal and colonic tissues. In ileum, the villus

architecture was severely damaged and this eventually gave rise to protein-rich bloody luminal fluid, as the toxin gained access to the deeper tissues. In the colon, although colonocytes were removed, the basement membrane remained intact; this resulted in a tissue-localised haemorrhage and a watery ultrafiltered luminal fluid low in protein.

Returning to the role of toxin A in PMC, it was observed that continuous membrane-like structures were seen overlying intoxicated colonic tissue. Mitchell et al. (1987c) speculate that the comparatively low volume of watery diarrhoea associated with PMC could arise as a result of limited foci of C. difficile infection within which toxin A is synthesized and where it would be bound or occluded and from which it would not disseminate widely. Massive colonic involvement (sometimes seen at autopsy) with little or no immediate pre-history of diarrhoea could be due to the production (within many close or near confluent foci) of very large quantities of toxin A, the absorption of which would result in rapidly lethal effects as observed in several test species when toxin A is injected parenterally.

This work seems potentially to provide much insight into the role of toxin A in disease. It was interesting to note that although toxin B produced a much greater effect on protein synthetic capabilities on tissue culture cells

it produced no such effect when administered into the intestinal lumen. This may well explain why for so long researchers have been unable to agree upon the correlation of cytopathic effect (produced mainly by toxin B) with disease severity. The cytopathic effect seen *in vitro* may be of little or no relevance *in vivo*. It also underlines how one needs to be extremely cautious in interpreting data obtained by use of cell lines.

One study by Aronsson *et al*. (1985b) which used an ELISA technique to assess levels of toxin A in stools from patients with AAD and AAC showed that 62 of 189 (33%) of these patients had detectable toxin A (i.e. present at 5ng/ml or greater). Considering the likely role of this toxin in causing tissue damage it might be expected that it would be detected in a higher percentage of individuals. Lyerly *et al*. (1983) detected toxin A in 17 of 29 (59%) of patients positive for *C. difficile* cytotoxin in stools.

Several reasons can be put forward for these low levels of toxin A detected in clinical specimens. Firstly, the toxin may be there at levels below that possible to detect with these ELISA techniques. Reduced sensitivity may also be due to interfering factors like stool proteases, which Viscidi *et al*. (1984) suggested may degrade the solid phase proteins (i.e. antibody) coating the ELISA plates.

In conclusion, it can be seen that there is still much

worthwhile research to be done on C. difficile. It may in fact be not very long before the mechanisms by which the organism elicits its pathogenic effects are uncovered. Once this is achieved it may then be simpler to assess what factors are important in determining why some individuals are more susceptible to developing C. difficile-associated disease than others.

BIBLIOGRAPHY

AL-JUMAILI,I.J. and BINT,A.J. (1981). Simple method of isolation and presumptive identification of Clostridium difficile. Zentralblatt fur Bacteriologie,Mikrobiologie und Hygiene, Series A, 250, 142-146.

AL-JUMAILI,I.J., SHIBLEY,M., LISHMAN,A.H. et al. (1984). Incidence and origin of Clostridium difficile in neonates. Journal of Clinical Microbiology, 19, 77-78.

ALY,R., SHINEFIELD,H.R., LITZ,C. et al. (1980). Role of teichoic acid in the binding of Staphylococcus aureus to nasal epithelial cells. Journal of Infectious Diseases, 141, 463-465.

AMBROSE,N.S., JOHNSON, M., BURDON,D,W. et al. (1985). The influence of single dose intravenous antibiotics on faecal flora and emergence of Clostridium difficile. Journal of Antimicrobial Chemotherapy, 15, 319-326.

APPELBAUM,P.C., KAUFMANN,C.S., KEIFER,J.C. et al. (1983). Comparison of three methods for anaerobe identification. Journal of Clinical Microbiology, 18, 614-621.

ARCHIBALD,A.R., COAPES,H.E. and STAFFORD,G.H. (1969). The action of dilute alkali on bacterial cell walls. Biochemical Journal, 113, 899-900.

ARNON,S.S., MILLS,D.C., DAY,P.A. et al. (1984). Rapid death of infant rhesus monkeys injected with Clostridium difficile toxins A and B: physiologic and pathologic basis. Journal of Pediatrics, 104, 34-40.

ARONSSON,B., GRANSTROM,M., MOLLBY,R. et al. (1983). Enzyme-linked immunosorbent assay (ELISA) for antibodies to Clostridium difficile toxins in patients with pseudomembranous colitis and antibiotic-associated diarrhoea. Journal of Immunological Methods, 60, 341-350.

ARONSSON,B., GRANSTROM,M., MOLLBY,R. et al. (1985a). Serum antibody response to Clostridium difficile toxins in patients with Clostridium difficile diarrhoea. Infection, 13, 97-101.

ARONSSON,B., GRANSTROM,M., MOLLBY,R. et al. (1985b). Enzyme immunoassay for detection of Clostridium difficile toxins A and B in patients with antibiotic-associated diarrhoea and colitis. European Journal of Microbiology, 4, 102-107.

ATTRIDGE,S.R. and ROWLEY,D. (1983). The specificity of Vibrio cholerae adherence and the significance of the slime agglutinin as a second mediator of in vitro attachment. Journal of Infectious Diseases, 147, 873-881.

BADDILEY,J. (1972). Teichoic acids in cell walls and membranes of bacteria. In Essays in Biochemistry. Eds P.N. Campbell and F.Dickens. Academic Press, London. pp35-77.

BANNO,Y., KUBAYASHI,T., KONO, H. et al. (1984). Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from Clostridium difficile. Reviews of Infectious Diseases, 6, supplement 1, S11-S20.

BANNO,Y., KUBAYASHI,T., WATANABE,K. (1981). Two toxins (D-1 and D-2) of Clostridium difficile causing antibiotic-associated colitis: purification and some characterization. Biochemistry International, 2, 629-635.

BARER,M.R. (1984). Adherence in anaerobes : an in vitro model of adhesion in Clostridium difficile. In Models of Anaerobic Infection - New Perspectives in Clinical Microbiology. Ed. M.J. Hill. Martinus Nijhoff, Dordrecht. pp201-211.

BARTLETT,J.G. (1979). Antibiotic-associated colitis. Clinics in Gastroenterology, 8, 783-801.

BARTLETT,J.G. (1981a). Antibiotic-associated pseudo-membranous colitis. Hospital Practice, 16, 85-95.

BARTLETT,J.G. (1981b). Antimicrobial agents implicated in Clostridium difficile toxin-associated diarrhoea or colitis. Johns Hopkins Medical Journal, 149, 6-9.

BARTLETT,J.G., CHANG,T.W., GURWITH, M. et al. (1978a). Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. New England Journal of Medicine, 298, 531-534.

BARTLETT,J.G., CHANG,T.W., MOON,N. et al. (1978b). Antibiotic-induced enterocolitis in hamsters: studies with eleven agents and evidence to support the pathogenic role of toxin-producing clostridia. American Journal of Veterinary Research, 39, 1525-1530.

BARTLETT,J.G. and LAUGHON,B. (1984). Clostridium difficile toxins. Microecology and Therapy, 14, 35-42.

BARTLETT,J.G., MOON,N., CHANG,T.W. et al. (1978c). Role of Clostridium difficile in antibiotic-associated pseudomembranous colitis. Gastroenterology, 75, 778-782.

BARTLETT,J.G., ONDERDONK,A.B., CISNEROS,R.L. et al. (1977). Clindamycin-associated colitis due to toxin-producing species of Clostridium in hamsters. Journal of Infectious Diseases, 136, 701-705.

BARTLETT,J.G., TAYLOR,N.S., CHANG,T.W. et al. (1980). Clinical and laboratory observations in Clostridium difficile colitis. American Journal of Clinical Nutrition, 33, 2521-2526.

BATE,G. (1986). Comparison of Minitek Anaerobe II, API An-Ident and RapID ANA systems for identification of Clostridium difficile. American Journal of Clinical Pathology, 85, 716-718.

BENDER,B.S., LAUGHON,B.E., GAYDOS,C. et al. (1986). Is Clostridium difficile endemic in chronic-care facilities? Lancet, II, 11-13.

BENNETT,G.C.J., ALLEN,E. and MILLARD, P.H. (1984). Clostridium difficile diarrhoea: a highly infectious organism. Age and Ageing, 13, 363-366.

BETZ,J.V. and ZEIKUS,J.G. (1968). Ultrastructure of the cell wall of Clostridium sporogenes. Bacteriological Proceedings. Abstracts of the 68th Annual Meeting of the American Society for Microbiology. Ann Arbor, Michigan. Abstract G16, p21.

BOLTON,R.P. (1982). Pseudomembranous colitis - disease, aetiology and treatment. European Journal of Chemotherapy and Antibiotics, 2, 115-122.

BOLTON,R.P., COTTER, K.L. and LOSOWSKY,M.S. (1985). Inhibition of neutrophil chemotaxis and phagocytosis by Clostridium difficile. Clinical Science, 68, supplement 11, p53.

BOLTON,R.P., SHERRIFF,R.J. and READ,A.E. (1980). Clostridium difficile-associated diarrhoea: a role in inflammatory bowel disease. Lancet, I, 383-384.

BOLTON,R.P., TAIT,S.K., DEAR,P.R.F. et al. (1984). Asymptomatic neonatal colonization by Clostridium difficile. Archives of Disease in Childhood, 59, 466-472.

BOLTON,R.P. and THOMAS,D.F.M. (1986). Pseudomembranous colitis in children and adults. British Journal of Hospital Medicine, 35, 37-42.

BOMMER,J., RITZ,E., ANDRASSY,K. et al. (1983). Effect of vaccination schedule and dialysis on hepatitis B vaccine response in uraemic patients. Proceedings of the European Dialysis and Transplant Association, 20, 161-166.

BORRIELLO,S.P. (1979). Clostridium difficile and its toxins in the gastrointestinal tract in health and disease. Research and Clinical Forums, 1, 33-35.

BORRIELLO,S.P. (1981) Gas-liquid chromatography and Clostridium difficile. Lancet, II, p1283.

BORRIELLO,S.P. and BARCLAY,F.E. (1985). Protection of hamsters against Clostridium difficile ileocecitis by prior colonisation with non-pathogenic strains. Journal of Medical Microbiology, 19, 339-350.

BORRIELLO,S.P., BARCLAY,F.E., REED,P.J. et al. (1987). Analysis of latex agglutination test for Clostridium difficile toxin A (D-1) and differentiation between Clostridium difficile toxins A and B and latex reactive protein. Journal of Clinical Pathology, 40, 573-580.

BORRIELLO,S.P. and HONOUR,P. (1981). Simplified procedure for the routine isolation of Clostridium difficile from faeces. Journal of Clinical Pathology., 34, 1124-1127.

BORRIELLO,S.P., HONOUR,P. and BARCLAY,F. (1982). Cross-infection and Clostridium difficile. Lancet, II, p661.

BORRIELLO,S.P. and LARSON,H.E. (1981). Antibiotic and pseudomembranous colitis. Journal of Antimicrobial Chemotherapy, 7, supplement A, S53-S62.

BOWMAN,R.A. and RILEY,T.V. (1986). Isolation of Clostridium difficile from stored specimens and comparative susceptibiltiy of various tissue culture cell lines to cytotoxin. FEMS Microbiology Letters, 34, 31-35.

BRETTLE,R.P., POXTON,I.R., MURDOCH,J.McC. et al. (1982). Clostridium difficile in association with sporadic diarrhoea. British Medical Journal, 284, 230-233.

BRETTLE,R.P. and WALLACE,E. (1982). Clostridium difficile from stools of normal children. Lancet, I, p1193.

BRETTLE,R.P. and WALLACE,E. (1984). Clostridium difficile- associated diarrhoea. Journal of Infection, 8, 123-128.

BROOKS,J.B. (1986). Review of frequency-pulsed electron-capture gas-liquid chromatography studies of diarrhoeal diseases caused by members of the family Enterobacteriaceae, Clostridium difficile and Rotavirus. Journal of Clinical Microbiology, 24, 687-691.

BROOKS,J.B., NUNEZ-MONTIEL,O.L., BASTA,M.T. et al. (1984a). Studies of stools from pseudomembranous colitis, rotaviral and other diarrhoeal syndromes by frequency-pulsed electron capture gas-liquid chromatography. Journal of Clinical Microbiology, 20, 549-560.

BROOKS,J.B., NUNEZ-MONTIEL,O.L., WYCOFF,B.J. et al. (1984b). Frequency-pulsed electron capture gas-liquid chromatographic analysis of metabolilites produced by Clostridium difficile in broth enriched with amino acids. Journal of Clinical Microbiology, 20, 539-548.

BROWN,R., COLLEE,J.G., POXTON,I.R. et al. (in press). Bacteroides, Fusobacterium and related organisms; anaerobic cocci; identification of anaerobes. In Mackie and McCartney. Practical Medical Microbiology. Ed. J.G. Collee et al. Churchill Livingstone, Edinburgh. Chapter 36.

BUCHANAN,A.G. (1984). Selective enrichment broth culture for detection of Clostridium difficile and associated cytotoxin. Journal of Clinical Microbiology, 20, 74-76.

BUGGY,B.P., HAWKINS,C.C. and FEKETY,R. (1985). Effect of adding sodium taurocholate to selective media on the recovery of Clostridium difficile from environmental surfaces. Journal of Clinical Microbiology, 21, 636-637.

BUGGY,B.P., WILSON,K.H. and FEKETY,R. (1983). Comparison of methods for recovery of Clostridium difficile from an environmental surface. Journal of Clinical Microbiology, 18, 348-352.

BURDON, D.W. (1982). Clostridium difficile: the epidemiology and prevention of hospital acquired-infection. Infection, 10, 203-204.

BURDON,D.W., GEORGE,R.H., MOGG,G.A.G. et al. (1981a). Faecal toxin and severity of antibiotic-associated pseudomembranous colitis. Journal of Clinical Pathology, 34, 548-551.

BURDON,D.W., THOMPSON,H., CANDY,D.C.A. et al. (1981b). Enterotoxin(s) of Clostridiium difficile. Lancet, II, 258-259.

BYRNE,M.D. (1982). The identification and subclassification of clostridia of clinical importance. Thesis presented for the Fellowship of the Institute of Medical Laboratory Sciences. Edinburgh University.

CARROLL,S.M., BOWMAN,R.A. and RILEY,T.V. (1983). A selective broth for Clostridium difficile. pathology, 15, 165-167.

CARRUTHERS,M.M. and KABAT,W.J. (1983). Mediation of staphylococcal adherence to mucosal cells by lipoteichoic acid. Infection and Immunity, 40, 444-446.

CASHORE,W.J., PETER,G., LAUERMANN,M. et al. (1981). Clostridia colonization and clostridial toxin in neonatal nectotizing enterocolitis. Journal of Pediatrics, 98, 308-311.

CATO,E.P., HASH,D.E., HOLDEMAN,L.V. et al. (1982). Electrophoretic study of Clostridium species. Journal of Clinical Microbiology, 15, 688-702.

CHANDLER,H.M. and GULASEKARAM,J. (1974). The protective antigen of a highly immunogenic strain of Clostridium chauvoei including an evaluation of its flagella as a protective antigen. Journal of General Microbiology, 84, 128-134.

CHANG,T.W. and ARESON,P. (1978). Neonatal necrotizing enterocolitis: absence of enteric bacterial toxins. New England Journal of Medicine, 299, p424.

CHANG,T.W., BARTLETT,J.G., GORBACH,S.L. et al. (1978). Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infection and Immunity, 20, 526-529.

CHANG,T.W. and GORBACH,S.L. (1982). Rapid identification of Clostridium difficile by toxin detection. Journal of Clinical Microbiology, 15, 465-467.

CHANG,T.W., LAUERMANN,M. and BARTLETT,J.G. (1979). Cytotoxicity assay in antibiotic-associated colitis. Journal of Infectious Diseases, 140, 765-770.

CHANG,T.W., SULLIVAN,M. and WILKINS,T.D. (1986). Insusceptibiltiy of foetal intestinal mucosa and foetal cells to Clostridium difficile toxins. Acta Pharmacologica Sinica, 7, 448-453.

CHEN,P.S., TORIBARA,T.Y. and WARNER,H. (1956). Microdetermination of phosphorus. Analytical Chemistry, 28, 1756-1758.

COLEY,J., DUCKWORTH,M. and BADDILEY,J. (1975). Extraction and purification of lipoteichoic acids from Gram-positive bacteria. Carbohydrate Research, 40, 41-52.

COLLART,F., TIELMANS,C., DRARWA,M. et al. (1983). Haemodialysis, continuous ambulatory peritoneal dialysis and cellular immunity. Proceedings of the European Dialysis and Transplant Association, 20, 190-194.

COLLEE,J.G. and AMYES,S.G.B. (1986). Relevance of bacterial resistance for clinical and community medicine : point of view of the bacteriologist. In Resistance and development of resistance. Proceedings of the BRL Kurhaus Workshop on Antibiotics. Eds M.F. Michel and J.W.M. van der Meer. Beecham Research Ltd. pp67-73.

COLLEE,J.G., WATT,B., FOWLER,E.B. et al. (1972). An evaluation of the GasPak system in the culture of anaerobic bacteria. Journal of Applied Bacteriology, 35, 71-82.

COLLIGNON,A., COTTE-LAFFITTE,J., QUERO,A.M. et al. (1986). Clostridium difficile and its cytotoxin in stools of hospitalized infants. Influence of antibiotic treatment. Pathologie Biologie, 34, 977-982.

COOK,H.C. (1972). Chapter 3. In Human Tissue Mucins. Ed. F.J. Baker. Butterworth and Co., London. pp10-23.

COOPERSTOCK,M., RIEGLE,L., FABACHER,D. et al. (1985). Relationship between faecal α-1 antitrypsin and colonization with Clostridium difficile in asymptomatic infants. Journal of Pediatrics, 107, 257-259.

COOPERSTOCK,M., RIEGLE,L., WOODRUFF,C.W. et al. (1983). Influence of age, sex and diet on asymptomatic colonization of infants with Clostridium difficile. Journal of Clinical Microbiology, 17, 830-833.

COOPERSTOCK,M.S., STEFFEN,E., YOLKEN.R. et al. (1982). Clostridium difficile in normal infants and sudden infant death syndrome: an association with infant formula feeding. Pediatrics, 70, 91-95.

CRAVEN,R.C. and MONTIE,T.C. (1981). Motility and chemotaxis of three strains of Pseudomonas aeruginosa used for virulence studies. Canadian Journal of Microbiology, 27, 458-460.

CRUICKSHANK,R., DUGUID,J.P., MARMION,B.P. (Eds) (1975). Medical Microbiology. 12th Edition, volume 2. Churchill Livingstone, Edinburgh. p122.

CUMMINS,A.J. (1961). Pseudomembranous enterocolitis and the pathology of nosology. American Journal of Digestive Diseases, 6, 429-431.

DEACON,A.G., DUERDEN,B.I. and HOLBROOK,W.P. (1978). Gas-liquid chromatographic analysis of metabolic products in the identification of Bacteroidaceae of clinical interest. Journal of Medical Microbiology, 11, 81-99.

DEARING,W.H., BAGGENSTOSS,A.H. and WEED,L.A. (1960). Studies on the relationship of Staphylococcus aureus to pseudomembranous enteritis and to postantibiotic enteritis. Gastroenterology, 38, p441-451.

DELMEE,M., BULLIARD,G. and SIMON,G. (1986). Application of a technique for serogrouping of Clostridium difficile in an outbreak of antibiotic-associated diarrhoea. Journal of Infection, 13, 5-9.

DELMEE,M., HOMEL,M. and WAUTERS,G. (1985). Serogrouping of Clostridium difficile strains by slide agglutination. Journal of Clinical Microbiology, 21, 323-327.

DELMEE,M. and MICHAUX,J.L. (1986). Prevention of Clostridium difficile outbreaks in hospitals. Lancet, II, p350.

DIMMIT,K. and SIMON,M. (1970). Antigenic nature of bacterial hook structures. Infection and Immunity, 1, 212-213.

DON,G.J. and DAVIS,A.E. (1981). The association between antibiotic-associated diarrhoea and Clostridium difficile toxin in children. Australian and New Zealand Journal of Medicine, 11, 433-434.

DONTA,S.T. and MYERS,M.G. (1982). Clostridium difficile toxin in asymptomatic neonates. Journal of Pediatrics, 100, 431-434.

DONTA,S.T., STUPPY,M.S. and MYERS,M.G. (1981). Neonatal antibiotic-associated colitis. American Journal of Diseases of Children, 135, 181-182.

DRAPKIN,M.S., WORTHINGTON,M.G., CHANG,T.W. et al. (1985). Clostridium difficile colitis mimicking acute peritonitis. Archives of Surgery, 120, 1321-1322.

DUBOIS,M., GILLES,K.A., HAMILTON,J.K. et al. (1956). Colorimetric method for the determination of sugars and related substances. Analytical Chemistry, 28, 350-356.

DZINK,J. and BARTLETT,J.G. (1980). In vitro susceptibility of Clostridium difficile isolates from patients with antibiotic-associated diarrhoea or colitis. Antimicrobial Agents and Chemotherapy, 17, 695-698.

EBRIGHT,J.R., FEKETY,R., SILVA,J. et al. (1981). Evaluation of eight cephalosporins in hamster colitis model. Antimicrobial Agents and Chemotherapy, 19, 980-986.

EDITORIAL. (1980). Clostridium difficile and chronic bowel disease. Lancet, I, 402-403.

ELLIOT,H,L., CARPENTER,C.C.J., SACK.R.B. et al. (1970). Small bowel morphology in experimental caniné cholera. Laboratory Investigation, 22, 112-120.

ELLIS,M.E., MANDAL,B.K., DUNBAR,E.M. et al. (1984). Clostridium difficile and its cytotoxin in infants admitted to hospital with infectious gastroenteritis. British Medical Journal, 288, 524-526.

ELLIS,M.E., WATSON,B.M., MILEWSKI,P.J. et al. (1983), Clostridium difficile colitis unassociated with antibiotic therapy. British Journal of Surgery, 70, 242-243.

ELSDEN,S.R., HILTON, M.G. and WALLER,J.M. (1976). The end products of the metabolism of aromatic amino acids by clostridia. Archives of Microbiology, 107, 283-288.

ELSTNER,C., LINDSAY,R., BOOK,L. et al. (1983). Clostridium difficile colonization following outpatient antibiotic use in children. Clinical Research, 31, Abstract 121.

FAIRWEATHER,S.D., GEORGE,R.H., KEIGHLEY,M.R.S. et al. (1980). Arthritis in pseudomembranous colitis associated with an antibody to Clostridium difficile toxin. Journal of the Royal Society of Medicine, 73, 524-525.

FALSEN,E., KAIJSER,B., NEHLS,L., et al. (1980). Clostridium difficile in relation to enteric bacterial pathogens. Journal of Clinical Microbiology, 12, 297-300.

FEKETY,R., KIM,K.H., BROWN,D., et al. (1981). Epidemiology of antibiotic-associated colitis. Isolation of Clostridium difficile from the hospital environment. American Journal of Medicine, 70, 906-908.

FINEGOLD,S.M., ATTEBERY,H.R. and SUTTER, V.L. (1974). Effect of diet on human faecal flora : comparison of Japanese and American diets. American Journal of Clinical Nutrition, 27, 1456-1469.

FINKEL,M.J. (1978). Magnitude of antibiotic use. Annals of Internal Medicine, 89, 791-792.

FRETER,R. (1981). Mechanisms of association of bacteria to mucosal surfaces. In Ciba Symposium 80. Adhesion and microorganism pathogenicity. Eds K. Elliot, M. O'Conner and J. Whelan. Pitman Medical Publishers, London. pp36-55.

FRETER,R. (1982). Bacterial association with the mucus gel system of the gut. In Microbiology. Ed. D. Schlessinger. American Society for Microbiology, Washington D.C. pp278-281.

FRETER,R., O'BRIEN,P.C.M. and HALSTEAD, J.A. (1978). Adhesion and chemotaxis as determinants of bacterial association with mucosal surfaces. In The Secretory Immune System and Caries Immunity. Eds J.R. McGhee and J. Mastecky. Plenum Press, New York. pp429-437.

GEORGE,R.H. (1986). The carrier state: Clostridium difficile. Journal of Antimicrobial Chemotherapy, 18, supplement A, S47-S58.

GEORGE,R,H. and HARTLEY,S (1983). Antibodies to Clostridium difficile cytotoxin in a pediatric population. American Journal of Diseases of Children, 137, p1024.

GEORGE,R.H., SYMONDS,J.M., DIMOCK,F. (1978a). Identification of Clostridium difficile as a cause of pseudomembranous colitis. British Medical Journal, I, p695.

GEORGE,W.L., KIRBY,B.D., SUTTER,V.L. et al. (1979a). Antimicrobial susceptibility of Clostridium difficile. In Microbiology. Ed. D. Schlessinger. American Society for Microbiology, Washington DC. pp 267-271.

GEORGE,W.L., ROLFE,R.D. and FINEGOLD,S.M. (1982). Clostridium difficile and its cytotoxin in faeces of patients with antimicrobial agent-associated diarrhoea and miscellaneous conditions. Journal of Clinical Microbiology, 15, 1049-1053.

GEORGE,W.L., ROLFE,R.D., MULLIGAN,M.E. et al. (1979b). Infectious diseases 1979 - antimicrobial agent-induced colitis: an update. Journal of Infectious Diseases, 140, 266-268.

GEORGE,W.L., ROLFE,R.D., SUTTER,V.L. et al. (1979c). Diarrhoea and colitis associated with antimicrobial therapy in man and animals. American Journal of Clinical Nutrition, 32, 251-257.

GEORGE,W.L., SUTTER,V.L., CITRON,F. et al. (1979d). Selective and differential medium for isolation of Clostridium difficile. Journal of Clinical Microbiology, 9, 214-219.

GEORGE,W.L., SUTTER,V.L. and FINEGOLD, S.M. (1977). Antimicrobial agent-induced diarrhoea - a bacterial disease. Journal of Infectious Diseases, 136, 822-828.

GEORGE,W.L., SUTTER,V.L. and FINEGOLD,S.M. (1978b). Toxigenicity and antimicrobial susceptibility of Clostridium difficile, a cause of antimicrobial agent-associated colitis. Current Microbiology, 1, 55-58.

GERDING,D.N., OLSON,M.M., PETERSON,L.R. et al. (1986). Clostridium difficile-associated diarrhoea and colitis in adults. Archives of Internal Medicine, 146, 95-100.

GIANFRILLI,P., PANTOSI,A. and LUZZI,I. (1985). Evaluation of gas-liquid chromatography for the rapid diagnosis of Clostridium difficile-associated disease. Journal of Clinical Pathology, 38, 690-693.

GOKAL,R., FRANCIS,D.M.A., GOODSHIP,T.H.J. et al. (1982). Peritonitis in continuous ambulatory peritoneal dialysis. Lancet, II, 1388-1391.

GORBACH,S.L., MENDA,K.B., THADEPALLI,H. et al. (1973). Anaerobic microflora of the cervix in healthy women. American Journal of Obstetrics and Gynaecology, 117, 1053-1055.

GREENFIELD,C., AGUILAR RAMIREZ,J.R., POUNDER,R.E. et al. (1983). Clostridium difficile and inflammatory bowel disease. Gut, 24, 713-717.

GREENFIELD,C., BURROUGHS,A., SZAWATHOWSKI,M. et al. (1981). Is pseudomembranous colitis infectious? Lancet, I, 371-372.

GUENTZEL,M.N. and BERRY,L.J. (1975). Motility as a virulence factor for Vibrio cholerae. Infection and Immunity, 11, 890-897.

GURIAN,L., WARD,T.T., and KATON,R.M. (1982). Possible foodborne transmission in a case of pseudomembranous colitis due to Clostridium difficile - influence of gastrointestinal secretions on Clostridium difficile infection. Gastroenterology, 83, 465-469.

GURWITH,M.J., RABIN,H.R., LOVE,K. et al. (1977). Diarrhoea associated with clindamycin and ampicillin therapy: preliminary results of a cooperative study. Journal of Infectious Diseases, 135, S104-S110.

HACHLER,H. and WUST,J. (1984). Reexamination by bacteriophage typing of Clostridium difficile strains isolated during a nosocomial outbreak. Journal of Clinical Microbiology, 20, p604.

HAFIZ,S., McENTEGART,M.G., MORTON,R,S. et al. (1975). Clostridium difficile in the urogenital tract of males and females. Lancet, I, 420-421.

HAFIZ,S. and OAKLEY,C.L. (1976). Clostridium difficile : isolation and characteristics. Journal of Medical Microbiology, 9, 129-136.

HAJI,T.C., LEWIS,P.S. and SANDERSON,P.J. (1981). Pseudomembranous colitis : an infectious disease? Lancet, II, 208-209.

HALE,H.W. and COSGRIFF, J.H. (1957). Pseudomembranous enterocolitis. American Journal of Surgery, 94, 710-717.

HALL,I.C. and O'TOOLE,E. (1935). Intestinal flora in new-born infants. American Journal of Diseases of Children, 49, 390-402.

HALL,S.M., CALVER,G.P. and WILLIAMS,M. (1985). A hospital outbreak of Clostridium difficile? Journal of Hospital Infection, 6, 312-322.

HARPOLD,D.J., WASILAUSKAS,B.L. and O'CONNOR,M.L. (1985). Rapid identification of Clostridium species by high-pressure liquid chromatography. Journal of Clinical Microbiology, 22, 962-967.

HASLAM,S.C., KETLEY,J.M., MITCHELL,T.J. et al. (1986). Growth of Clostridium difficile and production of toxins A and B in complex and defined media. Journal of Medical Microbiology, 21, 293-297.

HAWKINS,C.C., BUGGY,B.P., FEKETY,R. et al. (1984). Epidemiology of colitis induced by Clostridium difficile in hamsters: application of a bacteriophage and bacteriocin typing scheme. Journal of Infectious Diseases, 149, 775-780.

HEARD,S.R., O'FARRELL,S., HOLLAND,D. et al. (1986a). The epidemiology of Clostridium difficile with use of a typing scheme : nosocomial acquisition and cross infection among immunocompromised patients. Journal of Infectious Diseases, 153, 159-162.

HEARD,S.R., RASBURN,B., MATHEWS,R.C. et al. (1986b). Immunoblotting to demonstrate antigenic and immunogenic differences among nine standard strains of Clostridium difficile. Journal of Clinical Microbiology, 24, 384-387.

HOLDEMAN,L.V., CATO,E.P. and MOORE,W.E.C. (1977). Anaerobe Laboratory Manual, 4th Edition. Virginia Polytechnic Institute and State University, Blacksburg.

HOLDER,I.A., WHEELER,R. and MONTIE,T.C. (1982). Flagellar preparations from Pseudomonas aeruginosa : animal protection studies. Infection and Immunity, 35, 276-280.

HOLST,E., HELIN,I. and MARDH,P.A. (1981). Recovery of Clostridium difficile from children. Scandanavian Journal of Infectious Diseases, 13, 41-45.

HONDA,T., HERNADEZ,I., KATOH,T. et al. (1983). Stimulation of enterotoxin production of Clostridiium difficile by antibiotics. Lancet, I, p655.

HOWARD,J.M., SULLIVAN,S.N. and TROSTER,M. (1980). Spontaneous pseudomembranous colitis. British Medical Journal, 281, p356.

HUBERT,J.,IONESCO,H. and SEBALD,M. (1981). Detection de Clostridium difficile par isolement sur milieu minimal selectif et par immunofluorescence. Annales of Microbiology (Pasteur Institute), 132A, 149-157.

HUTCHEON,D.F., MILLIGAN,F.D., YARDLEY,J.H. et al. (1978). Cephalosporin-associated pseudomembranous colitis. Digestive Diseases, 23, 321-326.

HYAMS,J.S., BERMAN,M.M. and HELGASON,H. (1981). Nonantibiotic-associated enterocolitis caused by Clostridium difficile in an infant. Journal of Pediatrics, 99, 750-752.

JARVIS,W., NUNEZ-MONTIEL,O., THOMPSON,F. et al. (1983). Comparison of bacterial isolation ,cytotoxicity assay and counterimmunoelectrophoresis for detection of Clostridium difficile and its toxin. Journal of Infectious Diseases, 147, p778.

JESSOP,H.L. (1986). The immunochemistry of. Serratia marscesens. Thesis presented for Doctor of Philosophy, Aston University, Birmingham.

JONES,G.W. and RUTTER,J.M. (1972). Role of the K88 antigen in the pathogenisis of neonatal diarrhoea caused by Escherichia coli in piglets. Infection and Immunity, 6, 918-927.

JUSTUS,P.G., MARTIN,J.L., GOLDBERG,D.A. et al. (1982). Myoelectric effects of Clostridium difficile : motility-altering factors distinct from its cytotoxin and enterotoxin in rabbits. Gastroenterology, 83, 836-843.

KABINS, S.A. and SPIRA,T.J. (1975). Outbreak of clindamycin-associated colitis. Annales of Internal Medicine, 83, 830-831.

KAGAWA,H., OWARIBE,K., ASAKURA,S. et al. (1976). Flagellar hook protein from Salmonella SJ25. Journal of Bacteriology, 125, 68-73.

KAMLYA,S., NAKAMURA,S., YAMAKAWA,K. et al. (1986). Evaluation of a commercially available latex immunoagglutination test kit for detection of Clostridium difficile D-1 toxin. Microbiology and Immunology, 30, 177-181.

KAWATA,T., TAKEOKA,A., TAKUMI,K. et al. (1984). Demonstration and preliminary characterization of a regular array in the cell wall of Clostridium difficile. FEMS Microbiology Letters, 24, 323-328.

KEIGHLEY,M.R.B. (1983). Clostridium difficile and inflammatory bowel disease. Journal of Antimicrobial Chemotherapy, 11, 493-494.

KEIGHLEY,M.R.B., ALEXANDER-WILLIAMS,J., ARABI,Y. et al. (1978a). Diarrhoea and pseudomembranous colitis after gastrointestinal operations. Lancet, II, 1165-1167.

KEIGHLEY,M.R.B., BURDON,D.W., ARABI,Y. et al. (1978b). Randomized controlled trial of vancomycin for pseudomembranous colitis and postoperative diarrhoea. British Medical Journal, 2, 1667-1669.

KIM.K.H, DuPONT,H.L. and PICKERING,L.K. (1983). Outbreaks of diarrhoea associated with Clostridium difficile and its toxin in day-care centres: evidence of person-to-person spread. Journal of Pediatrics, 102, 376-382.

KIM,K.H., FEKETY,R., BATTS,D.H. et al. (1981). Isolation of Clostridium difficile from the environment and contacts of patients with antibiotic-associated colitis. Journal of Infectious Diseases, 143, 42-50.

KIM,K.H, FEKETY,R., BROWN,D. et al. (1980). Isolation of Clostridium difficile from the environment of patients with antibiotic-associated colitis. Clinical Research, 28, Abstract 372.

KIM,K., PICKERING,L.K., DUPONT,H.L. et al. (1984). In vitro and in vivo neutralizing activity of human colostrum and milk against purified toxins A and B of Clostridium difficile. Journal of Infectious Diseases, 150, 57-62.

KOPPLE,J.D. and JONES,M.R. (1979). Amino acid metabolism in patients with advanced uraemia and in patients undergoing chronic dialysis. Advanced Nephrology, 8, 133-268.

KRISHNAN,C. (1986). Detection of Clostridium difficile toxins by enzyme immunoassay. Journal of Hygiene, 96, 5-12.

KUDHAIR,A., DELMEE,M. and WAUTERS,G. (1986). Rapid identification of Clostridium difficile by determination of leucine arylamidase activity. European Journal of Clinical Microbiology, 5, 160-161.

KUIJPER,E.J., OUDBIER,J.H., STUIFBERGEN,W.N.H.M. et al. (1987). Application of whole-cell DNA restriction endonuclease profiles to the epidemiology of Clostridium difficile-induced diarrhoea. Journal of Clinical Microbiology, 25, 751-753.

LABREC,E.H., SCHNEIDER,H., MAGNANI,T.J. et al. (1964). Epithellial cell penetration as an essential step in the pathogenesis of bacillary dysentery. Journal of Bacteriology, 88, 1503-1518.

LAEMMLI,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

LAMBERT,P.A. and BOOTH,B.R. (1982). Exposure of outer membrane proteins on the surface of Pseudomonas aeruginosa PA01 revealed by labelling with [^{125}I] lactoperoxidase. FEMS Microbiology Letters, 14, 43-45.

LAMONT,J.T. and TRNKA, Y.M. (1980). Therapeutic implications of Clostridium difficile toxin during relapse of chronic inflammatory bowel disease. Lancet, I, 381-383.

LARSON,H.E. (1978). Antibiotic-induced, toxin-mediated, clostridial colitis in humans and hamsters. Clinical Research, 26, Abstract 322.

LARSON,H.E., BARCLAY,F.E., HONOUR,P. et al. (1982). Epidemiology of Clostridium difficile in infants. Journal of Infectious Diseases, 146, 727-733.

LARSON,H.E., PARRY,J.V., PRICE,A.B. et al. (1977). Undescribed toxin in pseudomembranous colitis. British Medical Journal, I, 1246-1248

LARSON,H.E., PRICE,A.B. and BORRIELLO,S.P. (1980). Epidemiology of experimental enterocolitis due to Clostridium difficile. Journal of Infectious Diseases, 142, 408-413.

LARSON,H.E., PRICE,A.B., HONOUR,P. et al. (1978). Clostridium difficile and the aetiology of pseudomembranous colitis. Lancet, I, 1063-1066.

LASHNER,B.A., TODORCZUK,J., SAHM,D.F. et al. (1985). Clostridium difficile culture-positive toxin-negative diarrhoea : a review of 45 cases. Gastroenterology, 88, p1465.

LAUGHON,B.E., VISCIDI,R.P., GDOVIN,S.L. et al. (1984). Enzyme immunoassays for detection of Clostridium difficile toxins A and B in faecal specimens. Journal of Infectious Diseases, 149, 781-788

LEE,A., O'ROURKE,J.L., BARRINGTON,P.J. et al. (1986). Mucus colonization as a determinant in intestinal infection by Campylobacter jejuni : a mouse caecal model. Infection and Immunity, 51, 536-546.

LEUNG.A.C.T., ORANGE,G., McLAY,A. et al. (1985). Clostridium difficile-associated colitis in uraemic patients. Clinical Nephrology, 24, 242-248.

LEVETT,P,N. (1984a). Detection of Clostridium difficile in faeces by direct gas liquid chromatography. Journal of Clinical Pathology, 37, 117-119.

LEVETT,P.N. (1984b). Use of enrichment cultures for the isolation of Clostridium difficile from stools. Microbios Letters, 25, 67-69.

LEVETT,P.N. (1984c). Clostridium difficile in the genital tract. British Journal of Venereal Diseases, 60, p276.

LEVETT, P.N. (1985a). Identification of Clostridium difficile using the API ZYM system. European Journal of Clinical Microbiology, 4, 505-507.

LEVETT,P.N. (1985b). Effect of antibiotic concentration in a selective medium on the isolation of Clostridium difficile from faecal specimens. Journal of Clinical Pathology, 38, 233-234.

LEVETT,P.N. (1986). Clostridium difficile in habitats other than the human gastro-intestinal tract. Journal of Infection, 12, 253-263.

LEVETT,P.N. and MARGARITIS-BASSOULIS,G. (1985). Evaluation of enrichment media for the isolation of Clostridium difficile from faeces. European Journal of Clinical Microbiology, 4, 135-136.

LEVINE,H.G., KENNEDY,M. and LAMONT,J.T. (1982). Counterimmunoelectrophoresis vs cytotoxicity assay for the detection of Clostridium difficile toxin. Journal of Infectious Diseases, 145, p398.

LIBBY,J.M., JORTNER,B.S. and WILKINS,T.D. (1982). Effects of the two toxins of Clostridium difficile in antibiotic-associated cecitis in hamsters. Infection and Immunity, 36, 822-829.

LIBBY,J.M. and WILKINS,T.D. (1982). Production of antitoxins to two toxins of Clostridium difficile and immunological comparison of the toxins by cross-neutralization studies. Infection and Immunity, 35, 374-376.

LISHMAN,A.H., AL JUMAILI,I.J., ELSHIBLY,E. et al. (1984). Clostridium difficile isolation in neonates in a special care unit. Lack of correlation with necrotizing enterocolitis. Scandanavian Journal of Gastroenterology, 19, 441-444.

LISHMAN,A.H., AL-JUMAILI,I.J. and RECORD,C.O. (1981a). Spectrum of antibiotic-associated diarrhoea. Gut, 22, 34-37.

LISHMAN,A.H., AL-JUMAILI,I.J. and RECORD,C.O. (1981b). Antitoxin production in antibiotic-associated colitis. Journal of Clinical Pathology, 34, 414-415.

LISTON,T.E. (1983). Clostridium difficile toxin associated with chronic diarrhoea and failure to gain weight. Clinical Pediatrics, 22, 458-460.

LONNROTH,I. and LANGE,S. (1983). Toxin A of Clostridium difficile: production, purification and effect in mouse intestine. Acta Pathologica Microbiologica et Immunologica Scandinavica, 91(B), 395-400.

LOWRY,O.H., ROSEBROUGH,N.J., FARR,A.L. et al. (1951). Protein measurement with the Folin phenol reagent. Biological Chemistry, 193, 265-275.

LUZZI,I., CAPRIOLI,A.,FALBO,V. et al. (1986). Detection of clostridial toxins in stools from children with diarrhoea. Journal of Medical Microbiology, 22, 29-31.

LYERLY,D.M., LOCKWOOD,D.E., RICHARDSON.S.H. et al. (1982). Biological activities of toxins A and B of Clostridium difficile. Infection and Immunity, 35, 1147-1150.

LYERLY,D.M., PHELPS,C.J. and WILKINS,T. (1985). Monoclonal and specific polyclonal antibodies for immunoassay of Clostridium difficile toxin A. Journal of Clinical Microbiology, 21, 12-14.

LYERLY,D.M., ROBERTS,M.D., PHELPS,C.J. et al. (1986). Purification and properties of toxins A and B of Clostridium difficile. FEMS Microbiology Letters, 33, 31-35.

LYERLY,D.M., SULLIVAN,N.M. and WILKINS,T.D. (1983). Enzyme-linked immunosorbent assay for Clostridium difficile toxin A. Journal of Clinical Microbiology, 17, 72-78.

LYERLY,D.M. and WILKINS,T.D. (1984). Characteristics of the toxins of Clostridium difficile. In Antibiotic-associated diarrhoea and colitis. Ed. S.P. Borriello. Martinus Nijhoff, Boston. pp90-102.

LYERLY,D.M. and WILKINS,T.D. (1986). Commercial latex test for Clostridium difficile toxin A does not detect toxin A. Journal of Clinical Microbiology, 23, 622-623.

MANDAL,B.K., WATSON,B. and ELLIS,M. (1982). Pseudomembranous colitis in a 5-week old infant. British Medical Journal, 284, 345-346.

MALAMOU-LADAS,H., O'FARRELL,S., NASH,J.Q. et al. (1983). Isolation of Clostridium difficile from patients and the environment of hospital wards. Journal of Clinical Pathology, 36, 88-92.

MALAMOU-LADAS,H., and TABAQCHALI,S. (1982). Inhibition of Clostridium difficile by faecal streptococci. Journal of Medical Microbiology, 15, 569-574.

MARIER,L., ALLEN,S. and SIDERS,J. (1984). Rapid enzymatic characterization of clinically encountered anaerobic bacteria with the API ZYM system. European Journal of Clinical Microbiology, 3, 294-300.

MARTIN,N.J., SIWASIN,J, and HOLDING.A.J. (1982). The bacterial population of a blanket peat. Journal of Applied Bacteriology, 53, 35-48.

MASFARI,A.N., KINGHORN,G.R. and DUERDEN,B.I. (1983). Anaerobes in genitourinary infections in men. British Journal of Venereal Diseases, 59, 255-259.

McNEELY,S.G., ANDERSON,G.D. and SIBAI,B.M. (1985). Clostridium difficile colitis associated with single-dose cefazolin prophylaxis. Obstetrics and Gynaecology, 66, 737-738.

MERIDA,V., MOERMAN,J., COLAERT,J. et al. (1986). Significance of Clostridium difficile and its cytotoxin in children. European Journal of Pediatrics, 144, 494-496.

MEYERS,S., MAYER,L., BOTTONE,E. et al. (1981). Occurance of Clostridium difficile toxin during the course of inflammatory bowel disease. Gastroenterology, 80, 697-700.

MIORNER,H., JOHANSSON,G. and KRONVALL, G. (1983). Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci. Infection and Immunity, 39, 336-343.

MITCHELL,T.J., KETLEY,J.M., BURDON,D.W. et al. (1987a). The effects of Clostridium difficile crude toxins and purified toxin A on stripped rabbit ileal mucosa in Ussing chambers. Journal of Medical Microbiology, 23, 199-204.

MITCHELL,T.J., KETLEY,J.M., BURDON,D,W. et al. (1987b). The effects of Clostridium difficile toxins A and B on membrane integrity and protein synthesis in intestinal cells in vivo and in vitro and in McCoy cells in vitro. Journal of Medical Microbiology, 23, 205-210.

MITCHELL,T.J., KETLEY,J.M., BURDON,D.W. et al. (1987c). Biological mode of action of Clostridium difficile toxin A: a novel enterotoxin. Journal of Medical Microbiology, 23, 211-219.

MITCHELL,T.J., KETLEY,J.M., HASLAM,S.C. et al. (1986). Effect of toxin A and B of Clostridium difficile on rabbit ileum and colon. Gut, 27, 78-85.

MOGG,G.A.G., KEIGHLEY,M.R.B., BURDON,D.W. et al. (1979). Antibiotic-associated colitis - a review of 66 cases. British Journal of Surgery, 66,738-742.

MONTIE,T.C., DOYLE-HUNTZINGER,D., CRAVEN,R.C. et al. (1982). Loss of virulence associated with absence of flagellum in an isogenic mutant of Pseudomonas aeruginosa in the burned-mouse model. Infection and Immunity, 38, 1296-1298.

MOON,H.W., WHIPP,S.C. and BAETZ,A.L. (1971). Comparative effects of enterotoxins from Escherichia coli and Vibrio cholerae on rabbit and swine small intestine. Laboratory Investigation, 25, 133-140.

MOORE.W.E.C., CATO,E.P. and HOLDEMAN,L.V. (1966). Fermentation patterns of some Clostridium species. International Journal of Systematic Bacteriology, 16, 383-415.

MORRIS,J.G., JARVIS,W.R., NUNEZ-MONTIEL,O.L. et al. (1984). Clostridium difficile colonisation and toxin production in a cohort of patients with malignant haematologic disorders. Archives of Internal Medicine, 144, 967-969.

MOSS,S. (1983). Isolation and identification of anaerobic organisms from the male and female urogenital tracts. British Journal of Venereal Diseases, 59, 182-185.

MULLER,E.L., PITT,H.A. and GEORGE,W.L. (1987). Prairie dog model for antimicrobial agent-induced Clostridium difficile diarrhoea. Infection and Immunity, 55, 198-200.

MULLIGAN,M.E., GEORGE,W.L., ROLFE,R.D. et al. (1980). Epidemiological aspects of Clostridium difficile-induced diarrhoea and colitis. American Journal of Clinical Nutrition, 33, 2533-2538.

MULLIGAN,M.E., HALEBIAN,S, KWOK,R.Y.Y. et al. (1986). Bacterial agglutination and polyacrylamide gel electrophoresis for typing Clostridium difficile. Journal of Infectious Diseases, 153, 267-271.

MULLIGAN,M.E., JEFFES,L.E., OISHI,J.S. et al. (1982). Selective broth medium for Clostridium difficile. Abstracts of the Annual Meeting of the American Society for Microbiology. Abstract C301. p321.

MULLIGAN,M.E., ROLFE,R.D., FINEGOLD,S.M. et al. (1979). Contamination of a hospital environment by Clostridium difficile. Current Microbiology, 3, 173-175.

NACHAMKIN,I., LOTZ-NOLAN,L. and SKALINA,D. (1986). Evaluation of a commercial cytotoxicity assay for detection of Clostridium difficile toxin. Journal of Clinical Microbiology, 23, 954-955.

NAKAMURA,S., MIKAWA,M., NAKASHIO,S. et al. (1981a). Isolation of Clostridium difficile from the faeces and the antibody in sera of young and elderly adults. Microbiology and Immunology, 25, 345-351.

NAKAMURA,S., SERIKAWA,T., MIKAWA,M. et al. (1981b). Agglutination, toxigenicity and sorbitol fermentation of Clostridium difficile. Microbiology and Immunology, 25, 863-870.

NASH,J.Q., CHATTOPADHYAY,B., HONEYCOMBE,J. et al. (1982). Clostridium difficile and cytotoxin in routine faecal specimens. Journal of Clinical Pathology, 35, 561-565.

NAVARRO-ALONSO,T., BERNABEU-ESCLAPEZ,A., VALERO-GUILLEN, P.L. et al. (1985). Identification of Clostridium difficile by detection of p-cresol in a cooked meat medium. European Journal of Clinical Microbiology, 4, 438-439.

NUNEZ-MONTIEL,O.L., THOMPSON,F.S. and DOWELL,V.R.Jr. (1983). Norleucine-tyrosine broth for rapid identification of Clostridium difficile by gas-liquid chromatography. Journal of Clinical Microbiology, 17, 382-385.

O'FARRELL,S., WILKS,M., NASH,J.Q. et al. (1984). A selective enrichment broth for the isolation of Clostridium difficile. Journal of Clinical Pathology, 37, 98-99.

OFEK,I. and BEACHEY,E.H. (1980). Bacterial adherence. Advances in Internal Medicine, 25, 503-532.

OFEK,I., BEACHEY,E.H., JEFFERSON,W. et al. (1975). Cell membrane-binding proteins of group A streptococcal lipoteichoic acid. Journal of Experimental Medicine, 141, 990-1003.

OISHI,J.S., MULLIGAN,M.E. and FINEGOLD,S.M. (1983). Failure to detect Clostridium difficile in foods. Journal of Infectious Diseases, 148, p360.

ONDERDONK,A.B., CISNEROS, R.L. and BARTLETT,J.G. (1980). Clostridium difficile in gnotobiotic mice. Infection and Immunity, 28, 277-282.

ONDERDONK,A.B., LOWE,B.R. and BARTLETT,J.G. (1979). Effect of environmental stress on Clostridium difficile toxin levels during continuous cultivation. Applied and Environmental Microbiology, 38, 637-641.

PANICHI,G., PANTOSI,A., GENTILE,G. et al. (1985). Clostridium difficile colitis in leukaemia patients. European Journal of Cancer and Clinical Oncology, 21, 1159-1163.

PARTON,R. (1975). Envelope proteins in Salmonella minnesota mutants. Journal of General Microbiology, 89, 113-123.

PASHBY,N.L., BOLTON,R.P. and SHERRIF,R.J. (1979). Oral metronidazole in Clostridium difficile colitis. British Medical Journal, 1, 1605-1606.

PEACH,S.L., BORRIELLO,S.P., GAYA,H. et al. (1986). Asymptomatic carriage of Clostridium difficile in patients with cystic fibrosis. Journal of Clinical Pathology, 39, 1013-1018.

PEARCE,C. and DINEEN,P. (1960). A study of pseudomembranous enterocolitis. American Journal of Surgery, 99, 292-300.

PEIKEN,S.R., GALDIBINI,J. and BARTLETT,J.G. (1980). Role of Clostridium difficile in a case of nonantibiotic-associated pseudomembranous colitis. Gastroenterology, 79, 948-951.

PEPERSACK,F., LABBE,M., NONHOFF,C. et al. (1983). Use of gas-liquid chromatography as a screening test for toxigenic Clostridium difficile in diarrhoeal stools. Journal of Clinical Pathology, 36, 1233-1236.

PETERSON,L.R., HOLTER,J.J., SHANHOLTZER,C.J. et al. (1986). Detection of Clostridium difficile toxins A (enterotoxin) and B (cytotoxin) in clinical specimens. Evaluation of a latex agglutination test. American Journal of Clinical Pathology, 86, 208-211

PHILLIPS,K.D., BRAZIER,J.S., LEVETT,P.N. (1985). Clostridia. In Isolation and identification of micro-organisms of medical and veterinary importance. Eds C.H. Collins and J.M. Grange. Academic Press, London. pp215-233.

PHILLIPS,K.D. and ROGERS,P.A. (1981). Rapid detection and presumptive identification of Clostridium difficile by p-cresol production on a selective medium. Journal of Clinical Pathology, 34, 642-644.

PIERCE,P.F.Jr., WILSON,R., SILVA,J. et al. (1982). Antibiotic-associated pseudomembranous colitis: an epidemiologic investigation of a cluster of cases. Journal of Infectious Diseases, 145, 269-274.

POTVLIEGE,C., LABBE,M. and YOURSSOWSKY,E. (1981) Gas-liquid chromatograhy as screening test for Clostridium difficile. Lancet, II, p1105.

POXTON,I.R. (1979). Serological identification of Bacteroides species by an enzyme-linked immunosorbent assay. Journal of Clinical Pathology, 32, 294-298.

POXTON,I.R. (1982). Detection and isolation of Clostridium difficile. European Journal of Chemotherapy and antibiotics, 2, 123-128.

POXTON,I.R. (1984). Demonstration of the common antigens of Clostridium botulinum, C. sporogenes and C. novyi by an enzyme-linked immunosorbent assay and electroblot transfer. Journal of General Microbiology, 130, 975-981.

POXTON.I.R., ARONSSON,B., MOLLBY,R. et al. (1984). Immunochemical fingerprinting of Clostridium difficile strains isolated from an outbreak of antibiotic-associated diarrhoea. Journal of Medical Microbiology, 17, 317-324.

POXTON,I.R. and BROWN,R. (1979). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cell-surface proteins as an aid to the identification of the Bacteroides fragilis group. Journal of General Microbiology, 112, 211-217.

POXTON,I.R., BROWN,R. and COLLEE,J.G. (1982). Detection of species-specific and cross-reactive cell-surface antigens of Bacteroides species by an indirect enzyme-linked immunosorbent assay. Journal of Medical Microbiology, 15, 223-231.

POXTON,I.R. and BYRNE,M.D. (1981a) Detection of Clostridium difficile toxin by counterimmuno-electrophoresis: a note of caution. Journal of Clinical Microbiology, 14, p349.

POXTON,I.R. and BYRNE,M.D. (1981b). Immunological analysis of the EDTA-soluble antigens of Clostridium difficile and related species. Journal of General Microbiology, 122, 41-46.

POXTON,I.R. and CARTMILL,T.D.I. (1982). Immunochemistry of the cell-surface carbohydrate antigens of Clostridium difficile. Journal of General Microbiology, 128, 1365-1370.

POXTON,I.R. and SUTHERLAND,I.W. (1976). The butanol-soluble proteins of Klebsiella aerogenes. Microbios, 15, 93-103.

RAMIREZ-RONDA,C.H. (1974). Incidence of clindamycin-associated colitis. Annals of Internal Medicine, 81, p860.

RAMPLING,A., WARREN,R.E., BEVAN,P.C. et al. (1985). Clostridium difficile in heamatological malignancy. Journal of Clinical Pathology, 38, 445-451.

RAUTENBERG,P., STENDER,F. and ULLMANN,U. (1986). Detection of Clostridium difficile toxin A by immunoblotting. Zentralblatt fur Bacteriologie, Microbiologie und Hygiene Series A, 261, 29-42.

RAUTENBERG,P., STENDER,F. and ULLMANN,U. (1987). Monospecific antibodies for detection of Clostridium difficile enterotoxin. European Journal of Microbiology, 6, 62-64.

REDMOND,S.C., KETLEY,J.M., MITCHELL,T.J. et al. (1985). Detection of Clostridium difficile enterotoxin. In Isolation and identification of micro-organisms of medical and veterinary importance. Eds C.H. Collins and J.M. Grange. Academic Press, London. pp237-249.

REGH,J.E. and PAKES,S.P. (1982). Implications of Clostridium difficile and Clostridium perfringens iota toxins in experimental lincomycin-associated colitis of rabbits. Laboratory Animal Science, 32, 253-257.

REINER,L., SCHLESINGER,M.J. and MILLER,G.M. (1952). Pseudomembranous colitis following aureomycin and chloramphenicol. Archives of Pathology, 54, 39-67.

RENNIE,R.P., ELLIOT,J.M., NARDINI,M.A. et al. (1984). Criteria for detection of Clostridium difficile toxin production by counterimmunoelectrophoresis. Journal of Clinical Microbiology, 20, 923-926.

RICHARDSON,S.A.. ALCOCK,P.A. and GRAY,J. (1983). Clostridium difficile and its toxin in healthy neonates. British Medical Journal, 287, p878.

RICHARDSON,S.A., BROOKFIELD,D.S.K., FRENCH,T.A. et al. (1981). Pseudomembranous colitis in a 5-week-old infant. British Medical Journal, 283, p1510.

RIFKIN,G.D., FEKETY,R. and SILVA,J. (1978). Neutralization by Clostridium sordellii antitoxin of toxins implicated in clindamycin-induced cecitis in the hamster. Gastroenterology, 75, 422-424.

RILEY,T.V., WYMER,V., BAMFORD,V.W. et al. (1986). Clostridium difficile in general practice and community health Journal of Hygiene, 96, 13-17.

RITCHIE,D.B.C., JENNINGS,L.C., LYNN,K,L. et al. (1982). Clostridium difficile-associated colitis: cross infection in pre-disposed patients with renal failure. New Zealand Medical Journal, 95, 265-267.

ROGERS,H.J., PERKINS,H.R. and WARD,J.B. (1980). Microbial cell walls and membranes. 1st Edition. Chapman Hall, London. p10.

ROGERS,T.R., PETROU,M., LUCAS,C. et al. (1981). Spread of Clostridium difficile among patients receiving non-absorbable antibiotics for gut decontamination. British Medical Journal, 283, 408-409.

ROLFE,R.D., HELEBIAN,S. and FINEGOLD,S.M. (1981). Bacterial interference between Clostridium difficile and normal faecal flora. Journal of Infectious Diseases, 143, 470-475.

ROSENBERG,M., GUTNICK,D. and ROSENBERG,E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. FEMS Microbiology Letters, 9, 29-33.

ROTHMAN,S.W. (1981). Presence of Clostridium difficile toxin in guinea pigs with penicillin-associated colitis. Medical Microbiology and Immunology, 169, 187-196.

RYAN,R.W., KWASNIK,I and TILTON,R.C. (1980). Rapid detection of Clostridium difficile toxin in human faeces. Journal of Clinical Microbiology, 12, 776-779

RYAN,R.W., KWASNIK,I. and TILTON,R.C. (1983). Improved immunologic detection of Clostridium difficile antigen by counterimmunoelectrophoresis. Diagnostic Microbiology and Infectious Disease, 1, 59-63.

SANDS,M., YUNGBLUTH,M. and SOMMERS,H.M. (1983) The non-value of counterimmunoelectrophoresis for the direct rapid detection of Clostridium difficile toxin in stool filtrates. American Journal of Clinical Pathology, 79, 375-377.

SAVAGE,D.C. (1980). Colonization by and survival of pathogenic bacteria on intestinal mucosal surfaces. In Adsorption of Microorganisms to Surfaces. Eds G. Bitton and K.C. Marshall. John Willey, London. pp176-206.

SCHRANK,G.D. and VERWEY,W.F. (1976). Distribution of cholera organsims in experimental Vibrio cholerae infections: proposed mechanisms of pathogenisis and antibacterial immunity. Infection and Immunity, 13, 195-203.

SELL,T.L., SCHABERG,D.R. and FEKETY,F.R. (1983). Bacteriophage and bacteriocin typing scheme for Clostridium difficile. Journal of Clinical Bacteriology, 17, 1148-1152.

SHAHRABADI,M.S., BRYAN,L.E., GAFFNEY,D. et al. (1984). Latex agglutination test for detection of Clostridium difficile toxin in stool samples. Journal of Clinical Microbiology, 20, 339-341.

SHERERTZ,R.J. and SARUBBI,F.A. (1982). The prevalence of Clostridium difficile and toxin in a nursery population: a comparison between patients with necrotizing enterocolitis and an asymptomatic group. Journal of Pediatrics, 100, 435-439.

SHIBL,A.M. (1985). Effect of antibiotics on adherence of microorganisms to epithelial cell surfaces. Reviews of Infectious Diseases, 7, 51-65.

SILVA,J., FEKETY,R., WERK;C. et al. (1984). Inciting and etiologic agents of colitis. Reviews of Infectious Diseases, 6, supplement 1, S214-S221.

SLEYTR,V.B. (1975). Heterologous reattachment of regular arrays of glycoproteins on bacterial surfaces. Nature, 257, 400-402.

SMITH,H. (1978). The determinants of microbial pathogenicity. In Essays in Microbiology. Eds J.R.Norris and M.H.Richmond. John Wiley and Sons, Chichester. Chapter 13.

SNYDER,M.L. (1940). The normal faecal flora of infants between two weeks and one year of age. Journal of Infectious Diseases, 66, 1-16.

STARK,P.L. and LEE,A. (1982). Clostridia isolated from the faeces of infants during the first year of life. Journal of Pediatrics, 100, 362-365.

STARK,P.L., LEE,A. and PARSONAGE, B.D. (1982). Colonization of the large bowel by Clostridium difficile in healthy infants: quantitative study. Infection and Immunity, 35, 895-899.

STEVENSON,J.R. and STRONGER,K.A. (1979). Protective cellular antigen of Clostridium chauvoei. American Journal of Veterinary Research, 41, 650-653.

STOLL,B.J., NAHMIAS,A,J., WICKLIFFE,C. et al. (1980). Bacterial toxin and neonatal necrotizing enterocolitis. Journal of Pediatrics, 96, 114-115.

SULLIVAN,N.M., PELLETT,S. and WILKINS,T.D. (1982). Purification and characterization of toxins A and B of Clostridium difficile. Infection and Immunity, 35, 1032-1040.

SUTPHEN,J.L., GRAND,R.J., FLORES,A. et al. (1983). Chronic diarrhoea associated with Clostridium difficile in children. American Journal of Diseases of Children, 137, 275-278.

SVENDSON,P.J. (1973). Fused-rocket immunoelectrophoresis. Scandanavian Journal of Immunology, 2, Supplement 1, A manual for quantitative immunoelectrophoresis. 69-70.

SWAINSCOW,T.D.V. (1983). Statistics at square one. British Medical Association, London.

SWARTZBERG,J.E., MARESCA,R.M. and REMINGTON,J.S. (1977). Clinical study of gastrointestinal complications associated with cllindamycin therapy. Journal of Infectious Diseases, 135, S99-S103.

TABAQCHALI,S., HOLLAND,D., O'FARRELL,S. et al. (1984a). Typing scheme for Clostridium difficile: its application in clinical and epidemiological studies. Lancet, I, 935-938.

TABAQCHALI,S., O'FARRELL,S., NASH,J.Q. et al. (1984b). Vaginal carriage and neonatal acquisition of Clostridium difficile. Journal of Medical Microbiology, 18, 47-53.

TAKAGI,A., NAKAMURA,K. and VEDA,M. (1965). Electron microscope studies of the intracytoplasmic membrane system of Clostridium botulinum. Japanese Journal of Microbiology, 9, 131-143.

TAKUMI,K. and KAWATA,T. (1974). Isolation of a common cell wall antigen from proteolytic strains of Clostridium botulinum. Japanese Journal of Microbiology, 18, 85-90.

TAN,J., BAYNE,L.H. and McLEOD,P.J. (1979). Pseudomembranous colitis: a fatal case following prophylactic cephaloridine therapy. Journal of the American Medical Association, 242, 749-750.

TAYLOR,N.S., THORNE,G.M. and BARTLETT,J.G. (1981). Comparison of two toxins produced by Clostridium difficile. Infection and Immunity, 34, 1036-1043.

TEDESCO,F.J. (1982). Pseudomembranous colitis: pathogenesis and therapy. Medical Clinics of North America, 66, 655-664.

TEDESCO, F.J. (1984). Antibiotics associated with Clostridium difficile mediated diarrhoea and/or colitis. In Antibiotic Associated Diarrhoea and Colitis. Ed. S.P. Borriello. Martinus Nijhoff, Boston. pp3-8.

TEDESCO,F.J., BARTON,R.W. and ALPERS,D.H. (1974). Clindamycin-associated colitis a prospective study. Annals of Internal Medicine, 81, 429-433.

THIRKELL,D., THAKKER,B., HERRIOTT,A. et al. (1984). A screen for Clostridium difficile in the vagina. An outpatient study using and comparing selective media. Antonie Van Leeuwenhoek Journal of Microbiology, 50, 355-360.

THOMAS,D.F.M., FERNIE,D,S., BAYSTON,R. et al. (1984). Clostridial toxins in neonatal necrotising enterocolitis. Archives of Disease in Childhood, 59, 270-272.

THOMAS,D.F.M., FERNIE,D.S., BAYSTON,R. et al. (1985). Enterocolitis in Hirschsprung's disease: a controlled study of the etiologic role of Clostridium difficile. Journal of Pediatric Surgery, 21, 22-25.

THOMAS,D.F.M., MALONE.M., FERNIE,D.S. et al. (1982). Association between Clostridium difficile and enterocolitis in Hirschsprung's disease. Lancet, I, 78-79.

THOMPSON,C.M., GILLIGAN,P.H., FISHER,M.C. et al. (1983). Clostridium difficile cytotoxin in a pediatric population. American Journal of Diseases in Childhood, 137, 271-274.

THOMSON,G., CLARK,A.H., HARE,K. et al. (1981). Pseudomembranous colitis after treatment with metronidazole. British Medical Journal, 282, 864-865.

TOFFLER,R.B., PINGOUD,E.G. and BURRELL, M.L. (1978). Acute colitis related to penicillin derivatives. Lancet, II, 707-709.

TOWBIN,H., STAEHELIN,T. and GORDON,J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America, 76, 4350-4354.

TRNKA,Y.M. and LAMONT,J.T. (1981). Association of Clostridium difficile toxin with symptomatic relapse of chronic inflammatory bowel disease. Gastroenterology, 80, 693-696.

TRNKA,Y,M. and LAMONT,J.T. (1984). Clostridium difficile colitis. Advances in Internal Medicine, 29, 85-107.

TSAI,C.M. and FRASCH,C.E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry, 119, 115-119.

TVEDE,M. and WILLUMSEN,L. (1982). Clostridium difficile in patients with irritable bowel syndrome and ulcerative colitis. Lancet, I, p1124.

USHIJIMA,H., SHINOZAKI,T. and FUJII,R. (1985). Detection of Clostridium difficile enterotoxin in neonates by latex agglutination. Archives of Disease in Childhood, 60, 252-254

VARKI,N.M. and AQUINO,T.I. (1982). Isolation of Clostridium difficile from hospitalized patients without antibiotic-associated diarrhoea or colitis. Journal of Clinical Microbiology, 16, 659-662.

VAS,S.I. (1983). Microbiologic aspects of chronic ambulatory peritoneal dialysis. Kidney International, 23, 83-92.

VISCIDI,R.P. and BARTLETT,J.G. (1981). Antibiotic-associated pseudomembranous colitis in children. Pediatrics, 67, 381-386.

VISCIDI,R., LAUGHON,B.E., HANVANICH,M. et al. (1984). Improved enzyme immunoassay for the detection of antigens in faecal specimens. Investigation and correction of interfering factors. Journal of Immunological Methods, 67, 129-143.

VISCIDI,R., LAUGHON,B.E., YOLKEN,R. et al. (1983). Serum antibody response to toxins A and B of Clostridium difficile. Journal of Infectious Diseases, 148, 93-100.

VISCIDI,R., WILLEY,S. and BARTLETT,J.G. (1981). Isolation rates and toxigenic potential of Clostridium difficile isolates from various patient populations. Gastroenterology, 81, 5-9.

VITERI,A.L., PAXTON,H.H. and DYKE,W.P. (1974). The spectrum of lincomycin-clindamycin colitis. Gastroenterology, 66, 1137-1144.

VOSBECK,K., METT,H., HUBER,U. et al. (1982). Effects of low concentrations of antibiotics on Escherichia coli adhesion. Antimicrobial Agents and Chemotherapy, 21, 864-869.

WADSTROM,T., FARIS,A. and HJERTEN,S. (1980). Adhesion of enteropathic bacteria to hydrophobic surfaces. In Microbial Adhesion to Surfaces. Ed. R.W.Berkeley. Ellis Harwood, Chichester. pp537-540

WALD,A., MENDELOW,H. and BARTLETT,J.G. (1980). Non-antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. Annals of Internal Medicine, 92, 798-799.

WECKE,J., REINICKE,B. and SCHALLEHN,G. (1974). Remarkable differences in the ultrastructure of the cell wall of toxigenic clostridia. In Proceedings of the 8th International Congress of Electron Microscopy 2. Canberra, Australia. 644-645.

WEEKE,B. (1973a). Rocket immunoelectrophoresis. Scandanavian Journal of Immunology, 2, supplement 1, A manual for quantitative immunoelectrophoresis, 37-46.

WEEKE,B. (1973b). Crossed immunoelectrophoresis. Scandanavian Journal of Immunology, 2, supplement 1, A manual for quantitative immunoelectrophoresis, 47-56.

WELCH,A.R., BORRIELLO,S.P. and BARCLAY,F.E. (1985). Simplified procedure for tissue culture in routine detection of cytotoxins. Journal of Clinical Pathology, 38, 835-837.

WELCH,D.F., MENGE,S.K. and MATSEN,J.M. (1980). Identification of toxigenic Clostridium difficile by counterimmunoelectrophoresis. Journal of Clinical Microbiology, 11, 470-473.

WELKON,C.J., LONG,S.S., THOMSON,C.M. et al. (1985). Clostridium difficile in patients with cystic fibrosis. American Journal of Diseases of Children, 139, 805-808.

WEST,S.E.H. and WILKINS,T.D. (1982). Problems associated with counterimmunoelectrophoresis assays for detecting Clostridium difficile toxin. Journal of Clinical Microbiology, 15, 347-349.

WEXLER,H., MULLIGAN,M.E. and FINEGOLD,S.M. (1984). Polyacrylamide gel electrophoresis patterns produced by Clostridium difficile. Reviews of Infectious Diseases, 6, supplement 1. S229-S234.

WICKEN,A.J. and KNOX,K.W. (1975). Lipoteichoic acids - a new class of bacterial antigens. Science, 187, 161-1167.

WILLEY,S. and BARTLETT, J.G. (1979). Cultures for Clostridium difficile in stools containing a cytotoxin neutralized by Clostridium sordellii antitoxin. Journal of Clinical Microbiology, 10, 880-884.

WILSON,K.H. (1983). Efficiency of various bile salt preparations for stimulation of Clostridium difficile spore germination. Journal of Clinical Microbiology, 18, 1017-1019.

WILSON.K.H., KENNEDY,M.J. and FEKETY,F.R. (1982a). Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile. Journal of Clinical Microbiology, 15, 443-446.

WILSON,K.H., SILVA,J. and FEKETY,F.R. (1981). Suppression of Clostridium difficile by normal hamster caecal flora and prevention of antibiotic-associated cecitis. Infection and Immunity, 34, 626-628

WILSON,K.H., SILVA,J. and FEKETY,R. (1982b). Fluorescent-antibody test for detection of Clostridium difficile in stool specimens. Journal of Clinical Microbiology, 16, 464-468.

WOOD-HELIE,S.J., DALTON,H.P. and SHADONY, S. (1986). Hydrophobic and adherence properties of Clostridium difficile. European Journal of Clinical Microbiology, 5, 441-445.

WU,T.C. and FUNG,J.C. (1983). Evaluation of the usefulness of counterimmunoelectrophoresis for diagnosis of Clostridium difficile-associated colitis in clinical specimens. Journal of Clinical Microbiology, 17, 610-613.

WU,T.C. and GERSCH,S.M. (1986). Evaluation of a commercial kit for the routine detection of Clostridium difficile cytotoxin by tisse culture. Journal of Clinical Microbiology, 23, 792-793.

WU,T.C., McCARTHY,V.P. and GILL, V.J. (1983). Isolation rate and toxigenic potential of Clostridium difficile isolates from patients with cystic fibrosis. Journal of Infectious Diseases, 148, p176.

WUST,J., SULLIVAN,N.M., HARDEGGER,U. et al. (1982). Investigation of an outbreak of antibiotic-associated colitis by various typing methods. Journal of Clinical Microbiology, 16, 1096-1101.

YANCY,R.J., WILLIS,D.L. and BERRY,L.J. (1978). Role of motility in experimental cholera in adult rabbits. Infection and Immunity, 22, 387-392.

YANCY,R.J., WILLIS,D.L. and BERRY,L.J. (1979). Flagella-induced immunity against experimental cholera in adult rabbits. Infection and Immunity, 25, 220-228.

YOLKEN,R.H., WHITCOMB,L.S., MARIEN,G. et al. (1981). Enzyme immunoassay for the detection of Clostridium difficile antigen. Journal of Infectious Diseases, 144, p378.

APPENDICES

APPENDIX 1

CCFA medium (George et al., 1979d)

40g proteose peptone no. 2 (Difco Laboratories, Detroit, Michigan, USA)

1g	NaCl	
0.1g	$MgSO_4$	(I used 0.2g $MgSO_4.7H_2O$)
6g	fructose	(ICN Pharmaceuticals)
20g	agar	
3ml	1% solution of neutral red in ethanol	

Make up to 1000ml with distilled water.
Autoclave at 121°C and 15lb/in^2 for 15 min.

Once the above solution has cooled to 50°C add:

500µg/ml of D-cycloserine
16µg/ml of cefoxitin
5ml of egg yolk (Oxoid).

PPY medium (Deacon et al., 1978)

20g proteose peptone (Oxoid)
10g yeast extract (Difco)
5g NaCl

Make up to 1000ml with distilled water, adjust to pH 7·4 and autoclave as above.

Standard solutions for GLC

Volatile fatty acids:

0.01M acetic acid
0.01M propionic acid
0.01M iso-butyric acid
0.01M n-butyric acid
0.01M iso-valeric acid
0.01M n-valeric acid
0.01M iso-caproic acid
0.01M n-caproic acid

Non-volatile fatty acids:

0.04M lactic acid
0.01M succinic acid

APPENDIX 1 continued:

Krebs-Ringer Solution

NaCl	9.0g
KCl	0.46g
$CaCl_2$	0.37g
KH_2PO_4	0.21g
$MgSO_4.7H_2O$	0.38g

Dissolve the above chemicals in distilled water and make up to 1090ml and autoclave.

Just prior to use, add filter sterilized $NaHCO_3$ (1.36g dissolved in a minimum quantity of distilled water). Check that the pH is about 7.

APPENDIX 2

Buffers for SDS-PAGE

Electrode buffer

0.025M Tris
0.192M glycine
0.1% Sodium dodecyl sulphate (SDS)

Method:

Weigh out 6.057g Tris (hydroxymethyl) methylamine (BDH Analar), 28.827g glycine (BDH chromatographically homogeneous) and 2.0g SDS (BDH specially pure) into separate containers.

Dissolve Tris and glycine in approximately 1000ml of distilled water, adjust pH to 8.3 with 1M NaOH (BDH Analar).

Add SDS. After it dissolves make up volume to 2000ml with distilled water. Store at room temperature.

Stacking gel buffer

0.25M Tris-HCl, pH6.8
0.2% SDS

Method:

Into separate containers, weigh out 15.143g Tris (hydroxymethyl) methylamine (BDH Analar) and 1.0g SDS (BDH specially pure).

Dissolve the Tris in approximately 250ml of distilled water; adjust to pH6.8 with 1M HCl (BDH Analar).
Add SDS and when it has dissolved make up to 500ml with distilled water.

Store at room temperature.

Separating gel buffer (double strength)

0.75M Tris-HCl, pH8.8
0.2% SDS

Method:

Weigh out 90.855g Tris (hydroxymethyl) methylamine (BDH

APPENDIX 2 continued:

Analar and dissolve in approximately 500ml of distilled water; adjust to pH8.8 with 1M HCl (BDH Analar).

Add 2.0g SDS (BDH specially pure), dissolve and make up volume to 1000ml with distilled water. Filter through Whatman No. 1 paper.

Store at room temperature.

Buffers for RIE, FRIE and CIE

Electrode buffer

Solution 1:	barbital sodium	26.0g
	barbital (barbitone)	4.24g
	distilled water	2000ml
Solution 2:	glycine	112.4g
	Tris (not Analar grade)	90.4g
	distilled water	2000ml

Method:

Mix equal volumes of solutions 1 and 2 (final molarity is 0.187M).

Check that the pH is 8.8.

Store at 4°C

1% Agarose for immunoelectrophoresis

25ml CIE buffer (see above)
75ml distilled water
1.0g agarose (BDH)

Method:

Mix ingredients and dissolve by boiling; stir continuously. Add Triton X-100 (scintillation grade) to give 1% v/v.

Dispense in 15 ml volumes for 1st dimension CIE and into 3ml volumes for RIE and 2nd dimension CIE.

APPENDIX 3

Viable counts obtained from three plates in an experiment comparing recovery of C. difficile on CCFA and 1/2 CCFA and from alcohol treated and untreated faecal samples

Figures are expressed as organisms per gramme of faeces.

Sample	CCFA	1/2 CCFA
A	3.26×10^6	2.72×10^6
	2.56×10^6	2.56×10^6
	2.93×10^6	2.81×10^6
A*	4.80×10^6	5.76×10^6
	5.28×10^6	6.08×10^6
	5.60×10^6	7.04×10^6
B	2.72×10^7	2.78×10^7
	2.72×10^7	2.94×10^7
	2.61×10^7	2.94×10^7
B*	3.54×10^7	3.65×10^7
	3.20×10^7	3.49×10^7
	3.38×10^7	----------

*: figures for alcohol treated samples

Experiment A

Colony counts of C. difficile growing on CCFA after 48h incubation of plates in adhesion experiment

Time in C.d suspension	Wash 1	Wash 2	Wash 3	Wash 4	Homogenized gut	
	10^1	UD	UD	UD	10^1	10^2
2 sec	29	91	28	11	44	/
5 min	59	134	53	22	256	/
10 min	67	256	118	82	+++	51
15 min	101	296	184	141	+++	79
30 min	152	456	297	173	+++	141
60 min	207	389	330	204	+++	184

see over/

Experiment B

Colony counts of C. difficile growing on CCFA after 48h incubation of plates in adhesion experiment

Time in C.d suspension	Wash 1	Wash 2	Wash 3	Wash 4	Homogenized gut	
	10^1	UD	UD	UD	10^1	10^2
2 sec	62	47	42	20	58	/
5 min	81	91	65	33	275	/
10 min	94	130	75	50	+++	44
15 min	114	170	123	68	+++	69
30 min	180	400	143	73	+++	146
60 min	224	114	300	256	+++	198

In both these tables (experiments A and B) the following applies:

UD: undiluted
/: dilution not plated
+++: too many colonies to count

APPENDIX 3 continued:

Colony counts of B. fragilis growing on BA after 48h incubation of plates in adhesion experiment

Time in B.f suspension	Wash 1	Wash 2		Wash 3		Wash 4	Homogenized gut		
	10^1	UD	10^1	UD	10^1	UD	10^1	10^2	10^3
2 sec	124	44	/	13	/	12	16	/	/
5 min	152	84	/	28	/	25	/	/	14
10 min	174	147	/	54	/	51	/	/	26
15 min	183	+++	62	58	/	44	/	/	46
30 min	197	386	/	/	26	232	/	/	58
60 min	212	+++	48	232	/	199	/	/	64

UD: undiluted

/: only figures used to calculate the viable count are recorded.

+++: too many colonies to count.

APPENDIX 3 continued:

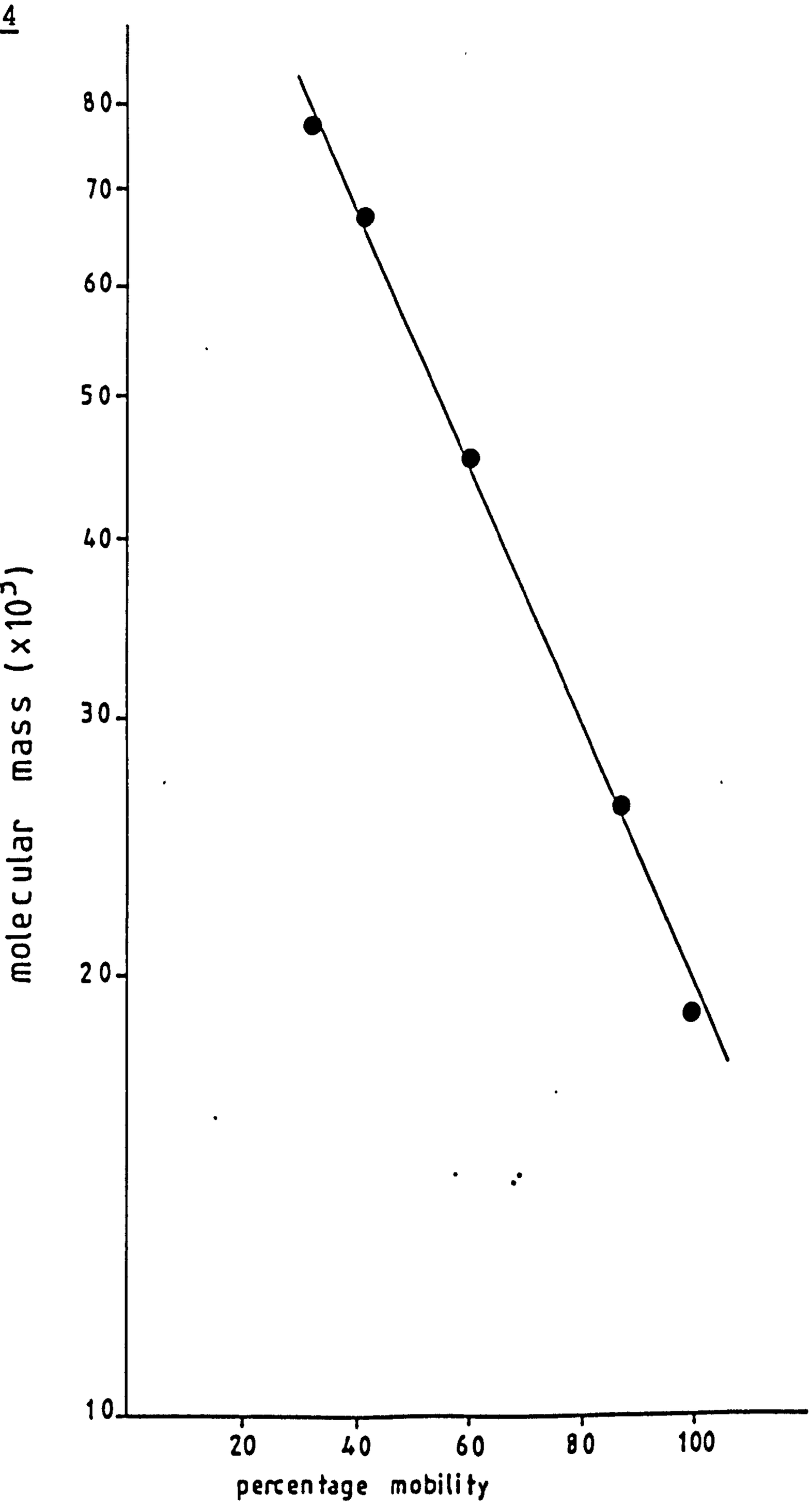
Colony counts obtained in experiment to assess the effect of prior treatment of ileum with B. fragilis on the association of C. difficile with ileal tissue

Organism	Time	Wash 1		Wash 2		Wash 3		Wash 4		Homogenized gut	
		10^2	10^3	10^1	10^2	UD	10^1	UD	10^1	10^1	10^2
B. fragilis	T_{15} recovery	333	/	175	/	/	132	/	110	/	56
B. fragilis	T_o control	190	/	98	/	197	/	118	/	301	/
B. fragilis	T_{15} control	/	36	/	33	/	136	/	88	/	80

		10^1	UD	UD	UD	10^1	10^2
C. difficile	T_{15} recovery	91	121	81	35	385	/
C. difficile	T_o control	354	41	29	40	46	/
C. difficile	T_{15} control	120	346	193	93	74	/

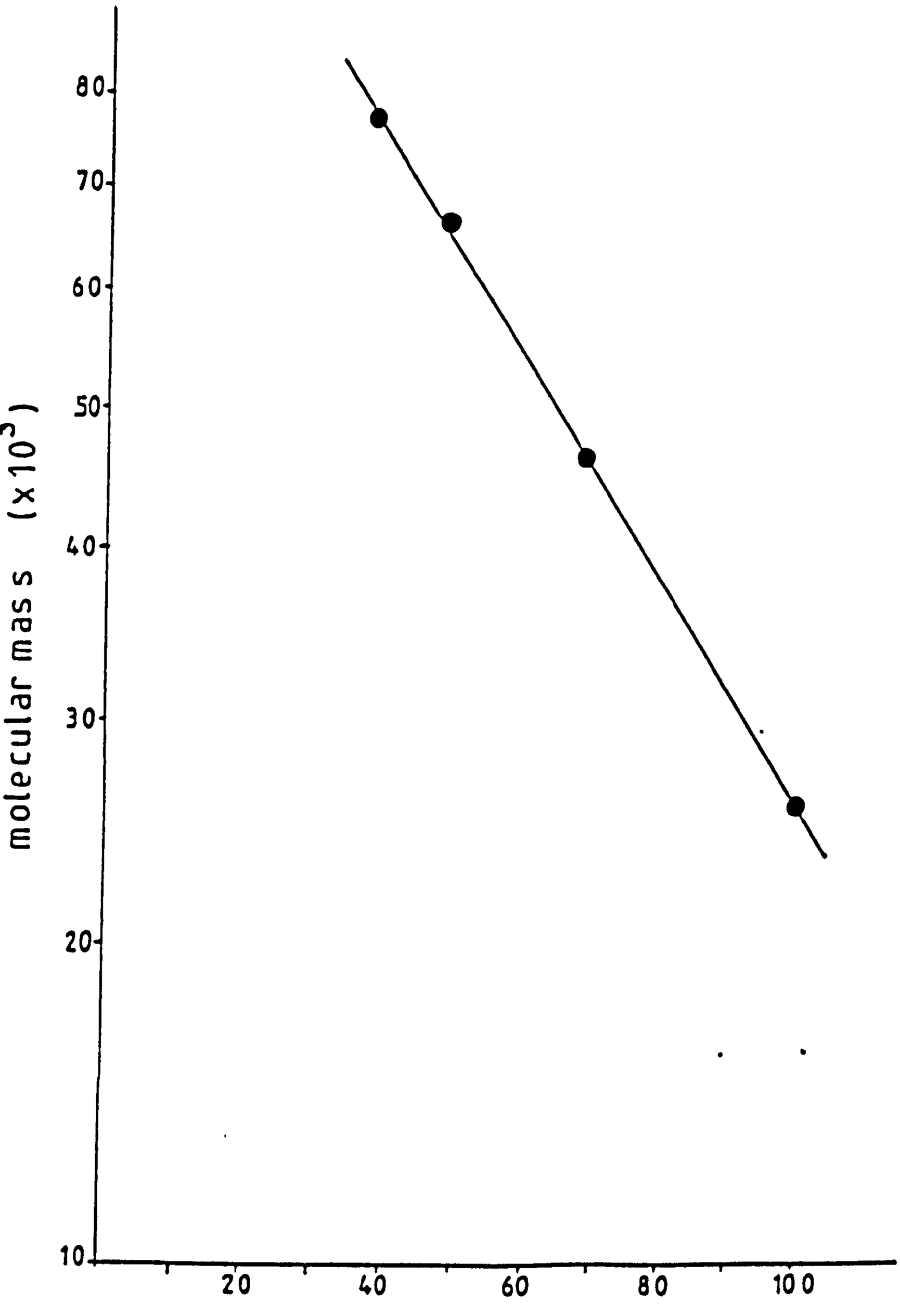
UD: undiluted
/: only figures used to calculate the viable count are recorded.

APPENDIX 4



Standard curve of molecular mass against percentage mobility for proteins separated during SDS-PAGE in Fig. 5.6.

APPENDIX 4 continued:



Standard curve of molecular mass against percentage mobility for proteins separated by SDS-PAGE (Heard et al. (1986b). Measurements were made from figures in this paper.

REPRINTS

Journal of Immunological Methods, 83 (1985) 241–248
Elsevier

JIM 03640

An Immunochemical Method for Fingerprinting *Clostridium difficile*

Jacqueline Sharp and Ian R. Poxton

Bacteriology Department, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

(Received 12 April 1985, accepted 18 June 1985)

The use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis in association with electrophoretic transfer of proteins to nitrocellulose and subsequent probing with antisera appears useful as a method for fingerprinting *Clostridium difficile*. Thorough testing of the stability of the antigenic nature of isolates of the organism during subculture and antigen preparation has shown it to be remarkably stable both in vitro and in vivo. Minor differences in the method of antigen extraction do not markedly alter the immunoblot patterns produced. It has also been demonstrated that an individual may harbour more than one strain of the organism at any one time. Results show the possible usefulness of this technique in studying the epidemiology of diarrhoeal disease known to be associated with *C. difficile*. It is suggested that for any serious study several colonies should be subcultured from the primary isolation plate.

Key words: *Clostridium difficile – immunoblotting – fingerprinting*

Introduction

Clostridium difficile is known to be associated with nearly all cases of antibiotic-associated pseudomembranous colitis. The organism also appears to be involved to some extent in a broad spectrum of other bowel conditions such as antibiotic-associated diarrhoea, post-operative diarrhoea and chronic inflammatory bowel disease. It has also been isolated from cases of sporadic non-antibiotic-associated diarrhoea (British Medical Journal, 1981; Brettle et al., 1982). Isolation of *C. difficile* from healthy adults is uncommon although the organism can be cultured from a high percentage of normal infants (George et al., 1977; Stark et al., 1982). The epidemiology of *C. difficile*-associated disease is poorly understood at present (Mulligan, 1984). There have been several reports indicating possible cross-infection between patients from whom the organism has subsequently been isolated. However lack of a reliable typing technique has hindered more detailed studies of these outbreaks. Sell et al. (1983) suggested that the use of bacteriophage could prove useful in typing of *C. difficile* while Tabaqchali et al. (1984) have used radiolabelling of growing cells as a possible means of studying the epidemiology of the organism. Since then Delmee

0022-1759/85/$03.30

et al. (1985) have shown that strains of the organism can be serogrouped into 6 different groups using a variety of antisera. Recently, in this laboratory the use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in association with electrophoretic transfer of proteins to nitrocellulose and subsequent probing with antisera has provided a useful method for fingerprinting the organism (Poxton et al., 1984). Further studies have subsequently been performed to test the reproducibility of results obtained with this technique. This paper provides a review of the experimental work carried out and offers guidelines for the successful application of the technique in the clinical situation.

Materials and Methods

Bacterial isolates

Isolation and identification of *C. difficile* was done as previously described (Brettle et al., 1982). Four strains of the organism, MPRL 558, 559, 589 and 720, which had been isolated from patients attending the Renal Unit in the Royal Infirmary of Edinburgh (RIE) during 1983 were used in this study. They were picked from the primary isolation plates as single colonies, cultured overnight in cooked meat broth (CMB) and lyophilised. MPRL 558 came from a severely ill patient; MPRL 559 from a patient with no diarrhoea while MPRL 589 and MPRL 720 came from 2 different patients with moderate diarrhoea.

Standard technique for culture and isolation of surface protein antigens

Lyophilised organisms were cultured in CMB for 17 h and 0.1 ml of this was inoculated into 100 ml proteose peptone yeast extract broth (PPY) supplemented with 0.04% (w/v) sodium carbonate and 0.075% (w/v) cysteine hydrochloride as described by Poxton et al. (1984). After 17 h incubation anaerobically at 37°C the cells were harvested (20,000 × *g* for 5 min at 4°C) and the washed pellet was resuspended in phosphate-buffered saline, pH 7.4, containing 10 mM EDTA and incubated at 45°C for 30 min. The supernatant, containing the antigens, was collected after 2 cycles of centrifugation at 10,000 × *g* for 2.5 min and used undialysed as antigen. The protein content of the preparation was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enrichment broth and selective agar

C. difficile was isolated from mouse faecal specimens using cycloserine-cefoxitin egg yolk agar (CCFA) (George et al., 1979). Enrichment was achieved using CCF broth which was CCFA without the egg yolk and agar.

SDS-PAGE

The buffer system of Laemmli (1970) with 10% slab gels as described by Poxton and Brown (1979) was used. EDTA extracts containing 25 μg of protein in 50 μl of sample buffer were run on gels in duplicate. One set of the separated proteins was stained with Coomassie blue and the other was used for transfer to nitrocellulose membrane.

Electroblot transfer

The method of Towbin et al. (1979) as described by Poxton et al. (1984) was followed. This involved transfer of the SDS-PAGE-separated EDTA extracts to nitrocellulose membrane in a Tris, glycine, methanol buffer pH 8.3 at 12 V and 40 mA for 18 h. After this the membrane was washed and probed with a rabbit antiserum raised against UV-killed cells of *C. difficile* NCTC 11223. The method of raising the antiserum was as described by Poxton and Byrne (1981).

Results

Work carried out with the standard technique for fingerprinting of *C. difficile* isolates which was described earlier (Poxton et al., 1984) has raised questions as to the reproducibility of the immunoblot patterns produced from one study to the next. Minor variations occurred between blotting patterns produced from different clinical isolates. It was important to find out whether these differences were due to real variations between strains or whether they arose simply because of differences in growth conditions of particular strains or variation in antigen extraction. These studies were therefore done to investigate the antigenic stability of isolates, both during culture of the organism and subsequent antigen extraction.

Effect of variation in phase of growth at harvesting

In the standard technique the organisms were cultured for 16–17 h before harvesting and antigen extraction. In order to find if culturing of the isolates for differing periods would produce differences in the resulting immunoblot patterns obtained, the following experiment was done. A 5% inoculum of MPRL 558 put into PPY medium was incubated anaerobically at 37°C and 100 ml amounts were harvested after 2, 4 and 8 h. Similarly a 1% inoculum was harvested after 16, 18, 20, 24 and 40 h. All other conditions remained the same throughout. It can be seen from the resulting immunoblot patterns produced (Fig. 1a) that there was a great degree of similarity in the banding patterns. There were minor variations, especially in the higher molecular weight polypeptides, but these may in part be due to slight variations in the amount of protein actually loaded onto the gel as seen in the corresponding Coomassie blue stain (Fig. 1b).

Variations in antigen extraction

Another likely source of variation between isolates could be differences in the actual method of antigen preparation. To investigate this 3 of the renal isolates were grown up in PPY for 17 h, harvested, washed and resuspended in EDTA buffer as described previously. The EDTA suspensions were then divided into 4 equal aliquots and each was subjected to one of the following treatments, (a) 'normal' antigen extraction at 45°C for 30 min, (b) extraction at 50°C for 30 min, (c) extraction at 45°C for 2.5 h, (d) freeze-thawing (−20°C to 37°C) 3 times over a period of 3 h and then 45°C for 30 min. These 4 EDTA suspensions were then centrifuged as described in Materials and Methods.

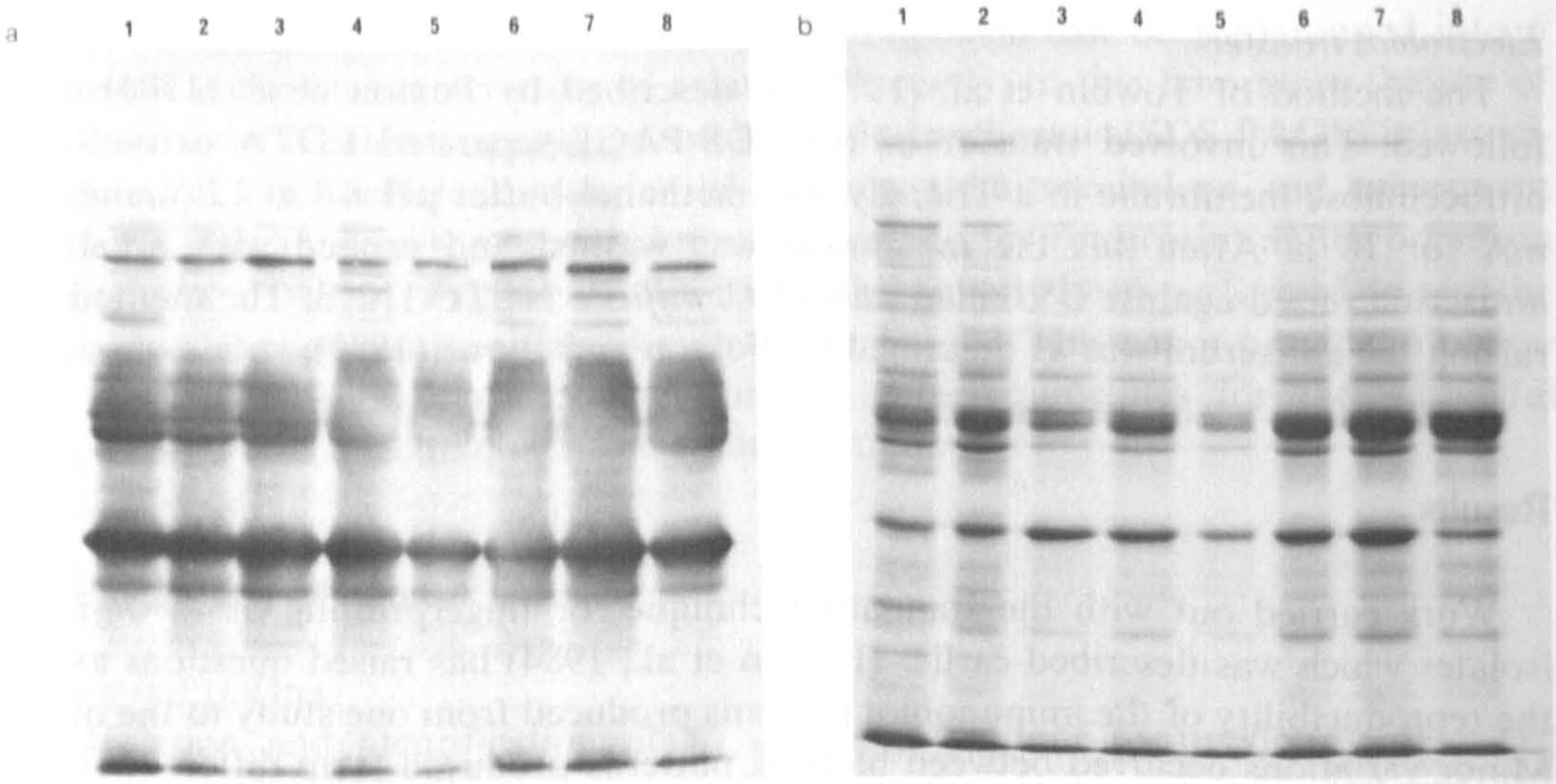


Fig. 1. (a) Immunoblot patterns produced by EDTA extracts of *C. difficile* MPRL 558 obtained 2–40 h after inoculation of PPY medium. Tracks 1–8 contained extracts obtained after 2, 4, 8, 16, 18, 20, 24 and 40 h respectively. Extracts were probed using antiserum raised to NCTC 11223. (b) Corresponding SDS-PAGE profile stained with Coomassie blue.

When these various extracts were run on gels it was found that MPRL 558 and 559 produced very similar banding patterns by Coomassie blue staining but there were consistent differences between the isolates in the patterns produced on immunoblotting (Fig. 2a and 2b). There was a much stronger reaction involving the high molecular weight proteins in MPRL 559 than occurred with MPRL 558 and this remained consistent despite the differing treatments of the extracts during extraction. However, MPRL 589 behaved differently. It was found that the extracts which had been heated for 2.5 h produced a blot pattern which was different from the others in the mid-region of the track (Fig. 2c). Why this occurred is not clear. With all 3 isolates the EDTA/freeze-thawed extracts had more protein bands apparent on the Coomassie stained gel. However, the immunoblot patterns obtained did not show up these additional bands indicating that they were probably intracellular proteins with which the antisera raised against whole UV-killed bacteria would not react.

Variations on subculture of the organisms

Having found that no major variations in immunoblot patterns could be accounted for by differences in culture or antigen preparation, further work was done to investigate the stability of an individual isolate both in vitro and in vivo. MPRL 558 was used for both these areas of investigation. A CMB culture of the isolate was passaged weekly into fresh pre-steamed CMB over the course of 7 weeks. An EDTA extract of each of these broths was prepared in the standard way after each had been incubated anaerobically at 37°C for 24 h. When these extracts were analysed by SDS-PAGE and immunoblotting the patterns produced were identical.

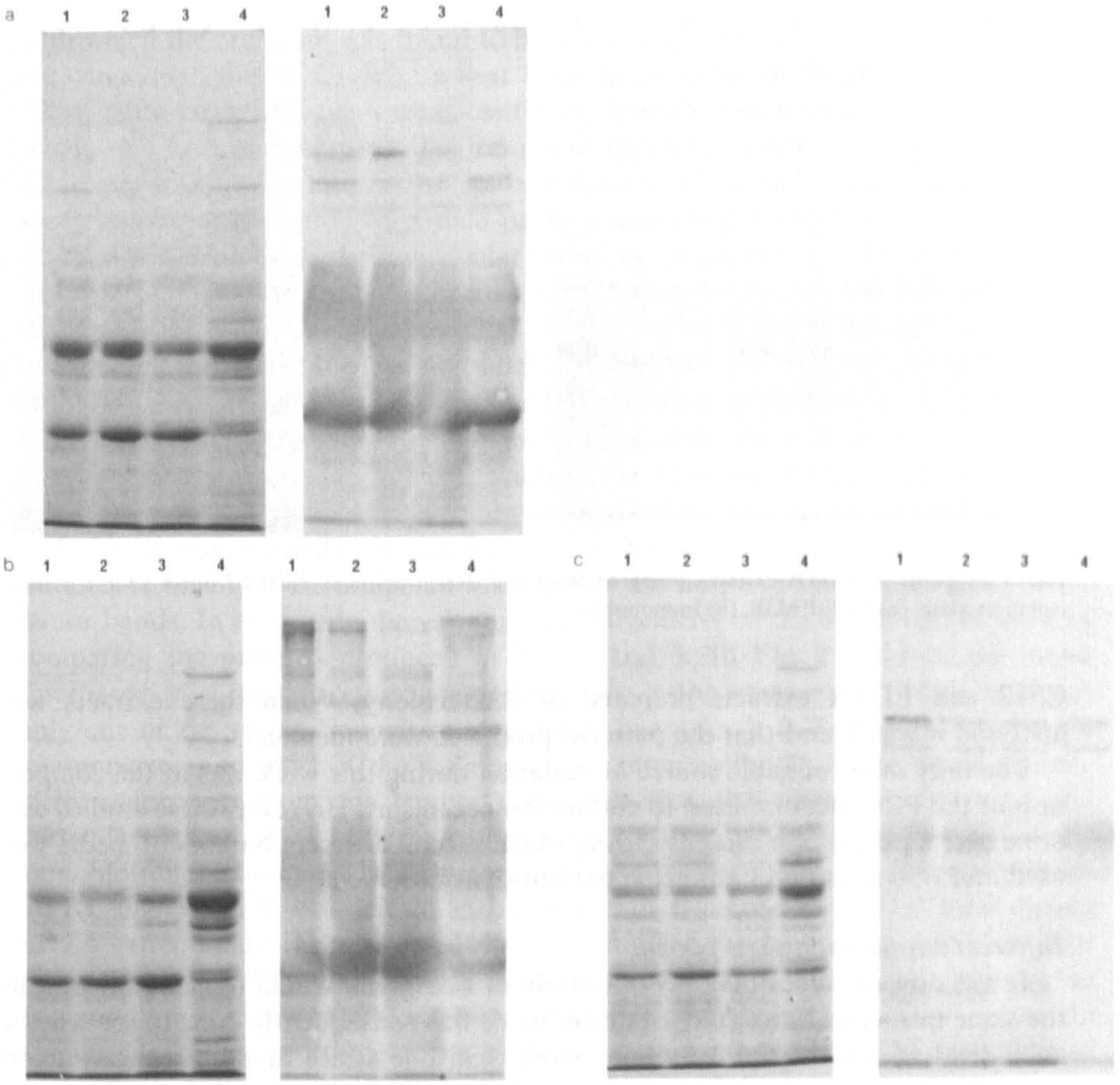


Fig. 2. SDS-PAGE and immunoblot patterns produced by (a) MPRL 558, (b) MPRL 559 and (c) MPRL 589 after various treatments during EDTA extraction. Track 1: extraction at 45°C for 30 min, track 2: 50°C for 2.5 h, track 3: 45°C for 2.5 h, track 4: freeze-thawed extract.

To study antigenic variation of the organism in vivo a spore suspension of MPRL 558 (100 μl saline inoculated with 10 *C. difficile* colonies from a 72 h blood agar plate) was given orally to a female BALB/c mouse which was known to be *C. difficile*-negative. Faecal samples were collected weekly from the animal over a period of 7 weeks. Each sample was inoculated into enrichment broth and the organisms were recovered by plating of these broths onto CCFA. The isolates were stored as CMB cultures and an EDTA extract of each isolate was prepared by the standard method. Again it was found that there were only minor variations between each of the isolates.

Another study was done with 3 strains where one colony from a CCFA plate was streaked onto blood agar. Eight colonies were subsequently picked from this into

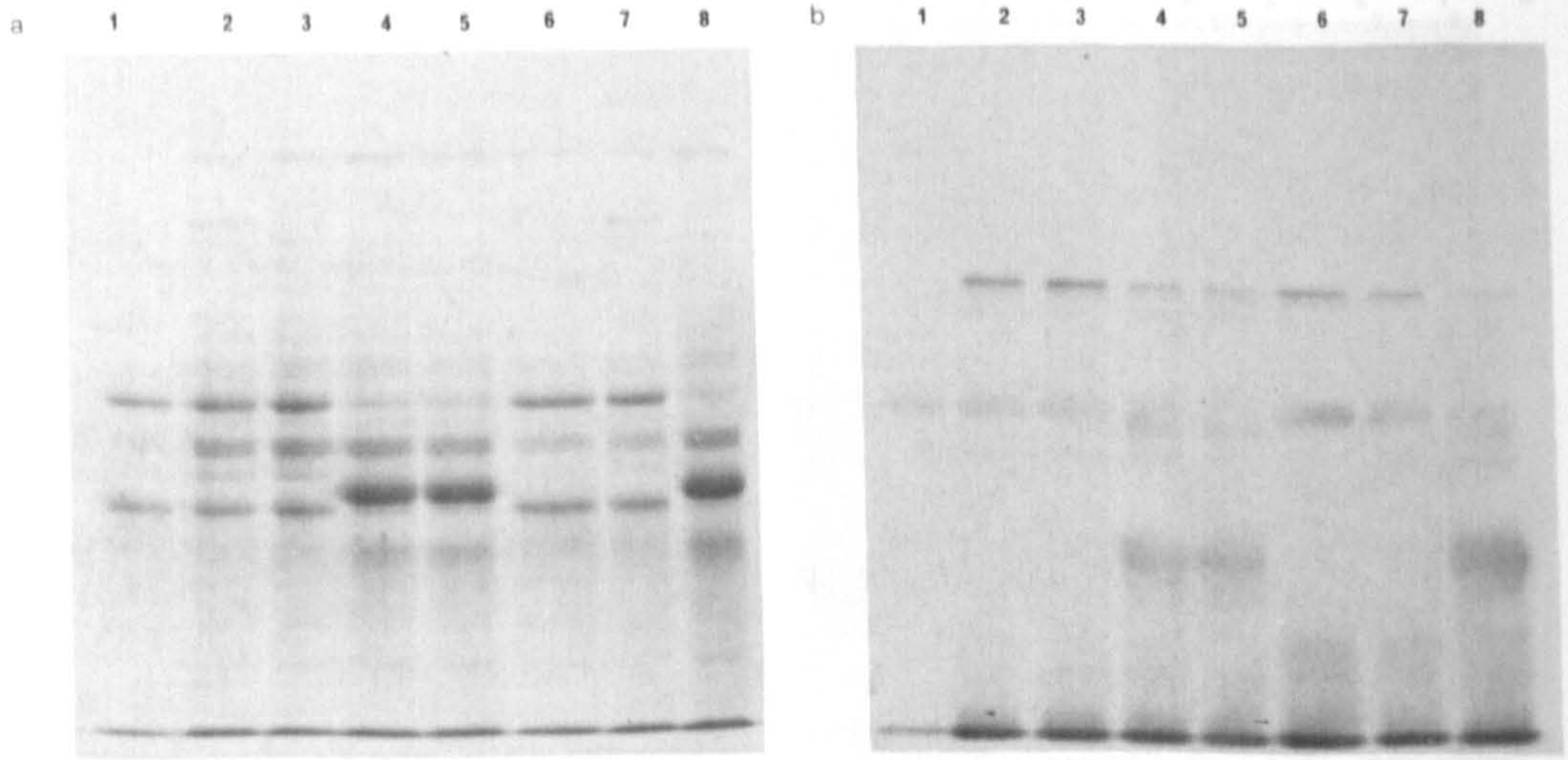


Fig. 3. Analysis of EDTA extracts prepared from 8 different colonies picked from a CCFA primary isolation plate. (a) SDS-PAGE, (b) immunoblot.

CMB and EDTA extracts prepared of each colony. When these extracts were analysed it was found that the patterns produced were identical.

The only other possible source of variation during this work was in the composition of the PPY medium used to culture the organisms. However, these studies were done over a period of 4 months during which several different batches of media were used and no variation in antigenic patterns was observed.

Different strains within one patient

It was now apparent that any one strain of *C. difficile* would remain antigenically the same throughout any study. Further work was consequently done to see whether an individual patient could harbour more than one strain of the organism at one time. The original faecal sample from which MPRL 720 had been isolated was spread onto CCFA. After incubation 8 different colonies were subcultured from this plate. EDTA extracts were prepared from all of these isolates which were subsequently analysed. It was found that from this primary isolation plate 2 different banding patterns were produced by the isolates (Fig. 3). The pattern produced in tracks 1, 2, 3, 6 and 7 was as expected for MPRL 720 (as indicated by other studies); the pattern in the other tracks corresponding to that of another isolate cultured from this patient 6 months previously. When this experiment was repeated with another 2 faecal samples one of these was also found to contain 2 different strains of *C. difficile*.

Discussion

The immunochemical fingerprinting of *C. difficile* strains described here has been used in this laboratory over the past 2 years to study various outbreaks of diarrhoea

with which the organism was found to be associated. The first investigation involved studying 28 strains of *C. difficile* sent from an outbreak in Sweden (Poxton et al., 1984). With this technique it was possible to show the existence of a common strain within one area of a hospital. The results of this study showed that this technique could prove useful in tracing the epidemiology of *C. difficile*. Since then a further study has been done involving renal patients attending the RIE who had differing degrees of diarrhoea. In this particular group of 18 patients 13 different strains of the organism appeared to be associated with their symptoms over a 9-month period (Cumming et al., unpublished results). It was found however that in one or two instances it was difficult to be absolutely certain whether or not 2 strains were the same or different. For this reason it was felt necessary to investigate the stability of the antigens that were being isolated and studied with this technique.

As we know from our previous studies, the Coomassie blue and immunoblot patterns vary considerably from strain to strain although one or two major bands and several minor bands are common to all strains. Some of the major bands in Coomassie blue gels are antigenic while others are not, and this is also true for the minor bands. In this study the variation in antigenicity of the bands can be seen by comparing the patterns produced in Fig. 1 and 3. In Fig. 1 most of the major Coomassie blue bands are also stained strongly in the immunoblot, while in Fig. 3 only one of the major Coomassie blue bands in tracks 1, 2, 3, 6 and 7 is strongly immunogenic and most of the staining of the immunoblot is due to minor bands.

It is apparent from the studies done here that the antigenic nature of any particular isolate of *C. difficile* will remain constant when analysed by this immunoblotting technique. The only major difference observed in an isolate was with MPRL 589 when it was left to incubate for an extended period of time during EDTA extraction of the antigens. It has been noticed that variations in the intensity of banding seen on the Coomassie stain can lead to difficulty in interpreting the immunoblotting patterns. Protein bands that are faint often do not show up on the immunoblot. However, these minor problems do not detract from the usefulness of this technique in the epidemiology of any outbreaks of diarrhoeal disease known to be associated with *C. difficile*.

Recommendations for successful use of immunoblotting

(1) Pick several colonies (at least 10) from the primary isolation plate in case there is more than one strain of *C. difficile* present in the patient.

(2) A standardised technique should be used for the culture and preparation of the EDTA antigen extracts. Cells should be incubated for 30 min and at a temperature of 45°C although minor variations in this do not appear to matter. Since different strains may vary in their sensitivity to changes during antigen extraction it is best to keep to a well-defined regimen. Freezing and thawing during the EDTA extraction procedure releases more protein: this complicates the Coomassie blue pattern, adds nothing to the immunoblot and should be avoided.

(3) Only compare EDTA antigens run on the same gel. It is difficult to compare one gel with another as it is impossible to reproduce running conditions accurately.

(4) When analysing the SDS-PAGE and immunoblot patterns produced by this

technique minor variations should be overlooked if the general picture arising is of similarity between the strains.

(5) Conclusions should be drawn using the information provided by both the gel and the immunoblot since some patterns that appear similar on the blot can appear quite different on the gel and vice versa. The advantage of doing an immunoblot as well as a Coomassie blue-stained gel is that the blot is simpler and easier to read.

Acknowledgements

This work was supported by a grant (K/MRS/50/C627) from the Scottish Home and Health Department.

We are grateful for the assistance given by Mr. R. Brown.

References

Brettle, R.P., I.R. Poxton, J. McC. Murdoch, R. Brown, M.D. Byrne and J.G. Collee, 1982, Br. Med. J. 284, 230.
British Medical Journal, 1981, Br. Med. J. 282, 1913.
Delmee, M., M. Homel and G. Wauters, 1985, J. Clin. Microbiol. 21, 323.
George, W.L., V.L. Sutter and S.M. Finegold, 1977, J., Infect. Dis. 136, 822.
George, W.L., V.L. Sutter, F. Citron and S.M. Finegold, 1979, J. Clin. Microbiol. 9, 214.
Laemmli, U.K., 1970, Nature (London) 227, 680.
Lowry, O.H., M.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, J. Biol. Chem. 193, 265.
Mulligan, M.E., 1984, Rev. Infect. Dis. 6 Suppl. 1, S222.
Poxton, I.R. and R. Brown, 1979, J. Gen. Microbiol. 112, 211.
Poxton, I.R. and M.D. Byrne, 1981, J. Gen. Microbiol. 122, 41.
Poxton, I.R., B. Aronsson, R. Mollby, C.E. Nord and J.G. Collee, 1984, J. Med. Microbiol. 17, 317.
Sell, T.L., D.R. Schaberg and F.R. Fekety, 1983, J. Clin. Microbiol. 17, 1148.
Stark, P.L., A. Lee and B.D. Parsonage, 1982, Infect. Immun. 35, 895.
Tabaqchali, S., D. Holland, S. O'Farrell and R. Silman, 1984, Lancet i, 935.
Towbin, H., T. Staehelin and J. Gordon, 1979, Proc. Natl. Acad. Sci. U.S.A. 76, 4350.

FEMS Microbiology Letters 34 (1986) 97–100
Published by Elsevier

FEM 02378

Analysis of the membrane lipocarbohydrate antigen of *Clostridium difficile* by polyacrylamide gel electrophoresis and immunoblotting

(*Clostridium difficile*, lipoteichoic acid, antigen, immunoblotting, silver staining)

Jacqueline Sharp and Ian R. Poxton

Bacteriology Department, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, U.K.

Received 4 November 1985
Revision received and accepted 22 November 1985

1. SUMMARY

The membrane lipocarbohydrate antigen (lipoteichoic acid analogue) of *Clostridium difficile* has been purified by aqueous phenol extraction and Sepharose 6B chromatography. After analysis by polyacrylamide gel electrophoresis (PAGE) and immunoblotting it has been shown to consist of a series of components of differing M_r. It appears as a regularly spaced ladder pattern similar to those shown for the lipopolysaccharide (LPS) of many Gram-negative bacteria.

2. INTRODUCTION

C. difficile is a Gram-positive, spore-forming, anaerobic bacterium and is well recognised as being the major cause of pseudomembranous and antibiotic-associated colitis [1]. The structure of the cell-surface appears to be typically Gram-positive: components analogous to wall and membrane teichoic acids have been demonstrated [2] and a regular surface array of protein subunits has been recognised [3]. The membrane teichoic acid analogue is not based on a poly-glycerol phosphate backbone as in most other Gram-positive bacteria so far investigated, but contains glucose, glucosamine, phosphate and fatty acid in the molar proportions of 2 : 1 : 1.6 : 0.04 [2].

In Gram-negative bacteria the LPS has been investigated by PAGE. After silver staining, it has been shown to be made up of a heterogeneous population of differing M_r, adjacent bands varying by one repeating unit of polysaccharide [4].

It might be expected that the appearance on PAGE of lipoteichoic acid molecules based on the typical poly-glycerol phosphate backbone would not show the characteristic ladder pattern of LPS. If the chain lengths were variable they would only differ by a single substituted glycerol phosphate unit, which might not be resolved on PAGE.

This present study uses PAGE and immunoblotting to investigate the immunochemistry of the atypical lipocarbohydrate of *C. difficile*. Antigenically related species are also compared.

3. MATERIALS AND METHODS

3.1. Culture of organisms and cell breakage

The strains used were *C. difficile* NCTC11223, and 3 clinical isolates, RIE1831, MPRL161 and MPRL597, *C. sordellii* NCTC8780 and *C. bifermentans* NCTC506.

A 0.1% (v/v) inoculum of an overnight culture

of bacteria in Robertsons' cooked meat broth was added to 6 l of pre-reduced proteose peptone yeast extract medium containing 0.04% (w/v) sodium carbonate and 0.075% (w/v) cysteine hydrochloride [5] and incubated anaerobically at 37°C for 18 h in an anaerobic cabinet (Forma Scientific). The bacteria were harvested (8500 × *g* for 10 min at 4°C) and broken as described previously [2]. After removal of the cell walls at 45 000 × *g* for 20 min, the supernate, containing the membrane antigen, was lyophilized prior to further extraction.

3.2. Extraction of membrane antigen

The lyophilized supernate was defatted with 2 × 200 ml chloroform/methanol (2 : 1, v/v) over 24 h and extracted with cold 80% (w/w) phenol as described by Coley et al. [6]. The membrane material (about 0.8 g) was suspended in distilled water to about 10% (w/v), mixed with an equal volume of 80% aqueous phenol (w/w) and stirred at room temperature for 30 min. This was centrifuged at 2500 × *g* for 20 min at 4°C. The upper aqueous layer, after dialysis, was mixed with an equal volume to 0.2 M acetic acid/acetate buffer, pH 5.0, containing 0.02 M $MgCl_2$. Ribonuclease and deoxyribonuclease (Sigma) were added and the mixture incubated under toluene at 37°C overnight. Phenol/water extraction was repeated, the upper aqueous layer dialysed and finally lyophilized. This was the crude membrane antigen.

Further purification of the MPRL161 sample (13 mg in 500 μl of distilled water) was carried out by fractionating on a Sepharose 6B column (30 × 1 cm) by the method of Coley et al. [6].

3.3. Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed by the procedure of Weeke [7] as described by Poxton and Byrne [8] with antiserum raised to MPRL161. Samples (10 μl) were applied at a concentration of 10 mg/ml in distilled water.

Fused rocket immunoelectrophoresis (FRIE) was done with antiserum raised to NCTC11223 as described by Svendsen [9] with the same buffer system as used for CIE. The pooled fractions were desalted on a 10 ml Sephadex G25 column.

3.4. Preparation of antiserum

Antisera against UV-killed, whole, washed cells of *C. difficile* NCTC11223 and MPRL161 were prepared as described previously [8].

3.5. PAGE and immunoblotting

The buffer system of Laemmli [10] with 10% slab gels as described by Poxton and Brown [11] with and without SDS was used. Samples (50 μl) containing 250 μg crude antigen or 25 μg purified antigen were run on each track. Gels were stained with silver [4] for 10–15 min. For immunoblotting gels without SDS were used. Materials were transferred to nitrocellulose membranes (0.2 μm pore, Sartorius) and treated with antiserum raised against NCTC11223 and anti-rabbit HRP conjugate, as previously described [12], except that Tween 20 was omitted from the washing solutions.

4. RESULTS

Crude aqueous phenol-extracted membrane antigens from 4 strains of *C. difficile* and one strain each of *C. sordellii* and *C. bifermentans* were analysed by SDS–PAGE and stained with silver. The results (Fig. 1) showed that the patterns produced by the *C. difficile* strains all appeared as a regularly spaced ladder pattern reminiscent of the pattern produced by the LPS of Gram-negative bacteria. The patterns produced by the other two species were indistinct, and even when more material was applied to the gel no ladder pattern could be demonstrated. Immunoblots of the *C. difficile* extracts with antiserum raised to *C. difficile* MPRL11223 revealed patterns of faint antigenic bands similar to those of the silver stain (results not shown).

One of the crude antigens (from *C. difficile* MPRL161) was analysed by CIE and produced a pattern identical to that previously produced by NCTC11223, i.e., two precipitin arcs showing partial identity [2]. After fractionation of the MPRL161 crude phenol extract on a Sepharose 6B column, two antigens were detected with FRIE, one eluting in the void volume, the other with a K_{av} of 0.71. This was similar to that previously shown for NCTC11223 [2]. The fractions corresponding to the two antigens were pooled, desalted and analysed further by PAGE and im-

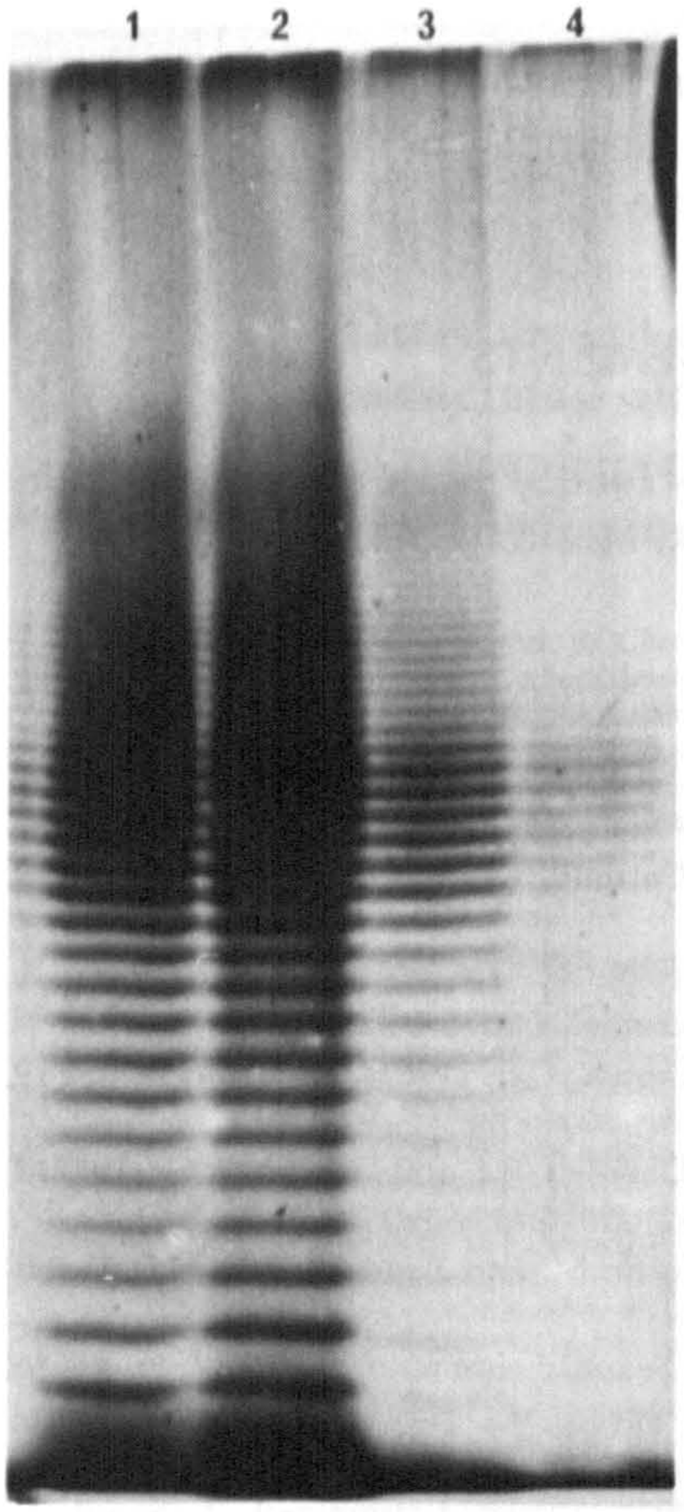


Fig. 1. Silver-stained SDS–PAGE of crude aqueous-phenol extracts of *C. difficile*. Lane 1, NCTC11223; lane 2, MPRL161; lane 3, RIE118311; lane 4, MPRL597. Samples (50 μl) containing 250 μg crude antigen were applied to each lane.

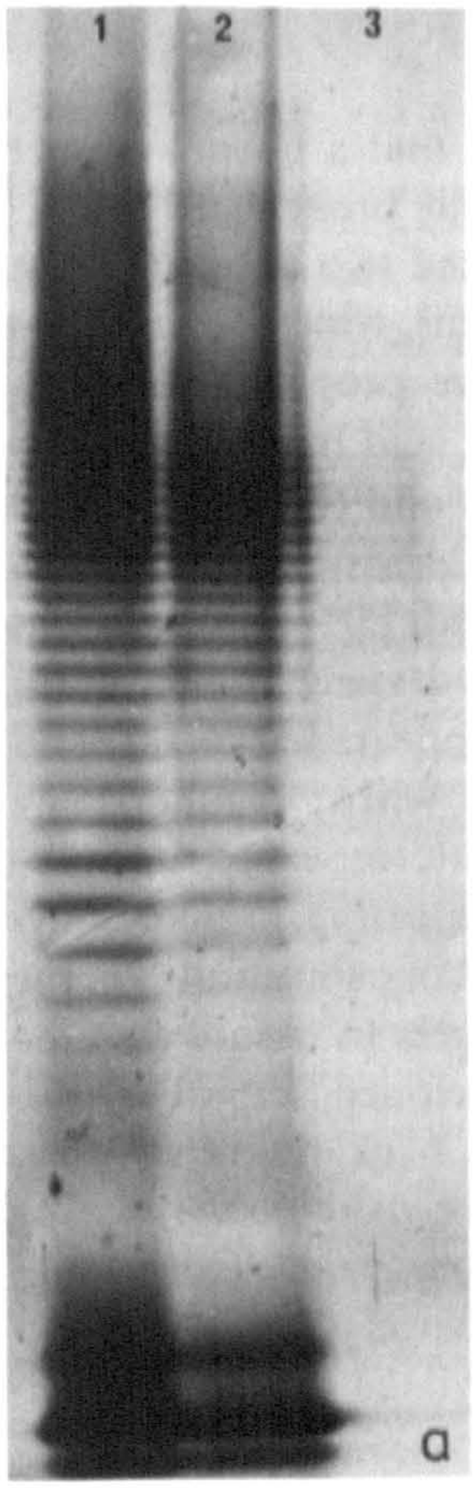


Fig. 2. (a) Silver-stained PAGE (without SDS) of crude aqueous-phenol extract of MPRL161 (lane 1) and the two antigenic fractions detected by FRIE from the Sepharose 6B column (lane 2 is first antigen and lane 3 the second). Lane 1 contained 250 μg crude antigen, and lanes 2 and 3 25 μg purified antigen. (b) Corresponding immunoblot with antiserum raised against NCTC11223.

munoblotting. Initial attempts to obtain good photographs of immunoblots were hampered by the apparent lack of sensitivity of the procedure. Subsequently, sodium dodecyl sulphate (SDS) was omitted from the gels but retained in the sample and electrode buffers. This resulted in greater sensitivity of detection by immunoblotting, and the silver-stained gel was extremely similar to the SDS-containing gel, except that the bands ran slower when SDS was omitted. The results of the PAGE and corresponding immunoblot of the crude membrane antigen and the two antigenic fractions after Sepharose 6B treatment are shown in Fig. 2. The material in fraction 1 gave an identical banding-pattern to that seen with the crude material. In the silver stain there was nothing apparent in the sample corresponding to fraction 2. In the immunoblot of fraction 2 an antigenic band was detected just behind the gel front.

Immunoblotting with antiserum to NCTC11223 of the crude extracts of the four *C. difficile* strains and the other two species was repeated, omitting SDS. The patterns produced by *C. difficile* were identical to those obtained with the silver stain. There was distinct cross-reaction of the *C. difficile* antiserum with the extracts from *Clostridium sordellii* and *Clostridium bifermentans*, part of the pattern in the mid-region of the *C. sordellii* and *C. bifermentans* gels being series of fine lines which appeared as a smear in the silver stain (not shown).

5. DISCUSSION

Previous work has shown that a phenol extract of lyophilized supernate from broken cells of *C. difficile* NCTC11223 contained two antigens when studied by CIE. These antigens, which appeared to be antigenically related, were proposed to correspond to (a) the lipoteichoic acid moiety found in all Gram positive organisms and (b) a deacylated form of this antigen which cross-reacts with the cell wall antigen (teichoic acid) [2]. In this present study the membrane carbohydrate has been shown to be extremely similar in appearance to the LPS of Gram-negative bacteria when examined by PAGE, giving a ladder pattern; each rung apparently differing by one repeating unit of polysaccharide. Relatively high concentrations of the antigen were applied to the gels to ensure that the silver-stained material developed rapidly, thus avoiding problems with high background staining. The identity of the second antigenic peak from the Sepharose 6B column is uncertain. This rather heterogeneous low-M_r material may be fragmented wall antigen or deacylated membrane antigen.

As far as we are aware this is the first report of the analysis of a lipoteichoic acid (or analogue) by PAGE. It has however been speculated that a ladder pattern seen in immunoblots of crude cell surface extracts of *Clostridium botulinum* might be due to such a molecule [13]. We do not know if this ladder pattern is produced by the LTA analogues of other clostridia or indeed of other Gram-positive bacteria.

ACKNOWLEDGEMENTS

This study was funded from a grant from the Scottish Home and Health department.

REFERENCES

[1] Bartlett, J.G. (1979) Rev. Infect. Dis. 1, 530–539.
[2] Poxton, I.R. and Cartmill, T.D.I. (1982) J. Gen. Microbiol. 128, 1365–1370.
[3] Kawata, T., Takeoka, A., Takumi, K. and Masuda, K. (1984) FEMS Microbiol. Lett. 24, 323–328.
[4] Tsai, C-M. and Frasch, C.E. (1982) Anal. Biochem. 119, 115–119.
[5] Holbrook, W.P., Duerden, B.I. and Deacon, A.G. (1977) J. Appl. Bacteriol. 42, 259–273.
[6] Coley, J., Duckworth, M. and Baddiley, J. (1975) Carbohydr. Res. 40, 41–52.
[7] Weeke, B. (1973) Scand. J. Immunol. 2 (Suppl. 1), 47–56.
[8] Poxton, I.R. and Byrne, M.D. (1981) J. Gen. Microbiol. 122, 41–46.
[9] Svendsen, P.J. (1973) Scand. J. Immunol. 2 (Suppl. 1), 69–70.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Poxton, I.R. and Brown, R. (1979) J. Gen. Microbiol. 112, 211–217.
[12] Poxton, I.R., Aronsson, B., Mollby, R., Nord, C.E. and Collee, J.G. (1984) J. Med. Microbiol. 17, 317–324.
[13] Poxton, I.R. (1984) J. Gen. Microbiol. 130, 975–981.

Reprinted from the BRITISH MEDICAL JOURNAL, *25th January 1986, 292, 238-239*

Diarrhoea due to Clostridium difficile associated with antibiotic treatment in patients receiving dialysis: the role of cross infection

Diarrhoea due to *Clostridium difficile* associated with treatment with antibiotics has been described among patients receiving peritoneal dialysis,[1] and cross infection is thought to be important.[2] We describe an outbreak of diarrhoea associated with *C difficile* in patients undergoing haemodialysis and continuous ambulatory peritoneal dialysis in which a "fingerprinting" technique of typing strains was used to investigate the possibility of person to person spread.

Patients, methods, and results

The table gives details of 18 patients from whom *C difficile* was isolated on stool culture. All developed diarrhoea while inpatients in the medical renal unit, Royal Infirmary, Edinburgh, between July 1983 and April 1984. *C difficile* had been isolated from only one patient with renal disease in the previous six months.

C difficile was cultured and identified as previously described[3]; strains were identified by the fingerprinting method of Poxton *et al*, using SDS-polyacrylamide gel electrophoresis of surface proteins extracted with edetic acid followed by Coomassie blue staining and an immunoblot probe using rabbit antiserum to cells of *C difficile* NCTC 11223 killed with ultraviolet light.[4] When *C difficile* was isolated patients were given oral vancomycin (500 mg every six hours) and other antibiotics were withdrawn if possible. Diarrhoea resolved in 12 patients. Four patients died during or shortly after treatment; all were severely debilitated by pre-existing medical conditions. The fingerprinting technique identified 13 different strains of *C difficile*. One strain occurred in five subjects (cases 12, 13, 14, 15, and 18) and one strain in two (cases 7 and 11); the 11 other strains occurred in only one patient each.

Comment

Cross infection with *C difficile* in hospitals has been clearly shown previously,[4] and seemed likely in this series of cases among our patients receiving dialysis; all had been inpatients in the medical renal unit, with considerable overlap in their periods of stay in hospital, and the rate of isolation of *C difficile* increased abruptly over 10 months. Standard measures to prevent spread of the organism were taken—namely, isolation when feasible, use of gown and gloves when working with patients, and careful attention to personal hygiene.

Isolation of patients was limited by lack of space and the specialised nursing that dialysis requires. The five patients from whom the same strain was isolated were probably cross infected; all were nursed in one of two adjacent cubicles, the first four within one month. The isolation of 13 different strains of *C difficile* appears, however, to exclude cross infection as the major mechanisms by which organisms were acquired during this outbreak. Among patients undergoing dialysis who have uraemia the frequent use of broad spectrum antibiotics, defective immunity, abnormal nutrition, and perhaps other changes in gut flora or mucosal defence mechanisms might combine to permit acquisition of *C difficile* or to promote its selective growth.[5] After this outbreak we tried to give as narrow a range of antibiotic treatment as possible and avoided oral antibiotics, particularly oral cephalosporins; the incidence of isolation of *C difficile* and related clinical disease returned to a low level.

We recommend early selective faecal culture for *C difficile* in any patients undergoing dialysis who have diarrhoea. Our findings suggest that cross infection with *C difficile* may occur in patients receiving dialysis, although it is not always the major mechanism of acquisition of this organism. It would be unwise to abandon standard measures against cross contamination, and it should be appreciated that patients undergoing dialysis may be particularly prone to infection with *C difficile*.

We thank the Scottish Home and Health Department and Upjohn Co for financial support; R Brown, A B Harris, and G Fleming for technical help; Professor J G Collee for advice and support; and Professor J S Robson, Dr Anne T Lambie, and Dr R J Winney for advice and permission to report on patients under their care.

1 Gokal R, Ramos JM, Francis DMA, *et al*. Peritonitis in continuous ambulatory peritoneal dialysis. Laboratory and clinical studies. *Lancet* 1982;ii:1388-91.
2 Ritchie DBC, Jennings LC, Lynn KL, Bailey RR, Cook HB. Clostridium difficile-associated colitis: cross-infection in predisposed patients with renal failure. *NZ Med J* 1982;95:263-7.
3 Poxton IR. Detection and isolation of Clostridium difficile. *European Journal of Chemotherapy and Antibiotics* 1982;2:123-8.
4 Poxton IR, Aronsson B, Mollby R, Nord CE, Collee JG. Immunochemical fingerprinting of Clostridium difficile strains isolated from an outbreak of antibiotic-associated colitis and diarrhoea. *J Med Microbiol* 1984;17:317-24.
5 Larson HE, Price AB, Honour P, Borriello SP. Clostridium difficile and the aetiology of pseudomembranous colitis. *Lancet* 1978;i:1063-6.

(Accepted 22 October 1985)

University Department of Medicine and Medical Renal Unit, Royal Infirmary, Edinburgh EH3 9YW
ALLAN D CUMMING, MB, MRCP, lecturer
BRIAN J THOMSON, MB, MRCP, registrar

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH8 9AG
JACQUELINE SHARP, BSC, research student
IAN R POXTON, BSC, PHD, lecturer
ANDREW G FRASER, BSC, MD, senior lecturer

Correspondence to: Dr Fraser.

Details of patients from whom C difficile was isolated

Case No	Age (years)	Sex	Type of dialysis	Type of infection	Antimicrobials given	Month when strain isolated	Outcome
1	61	F	CAPD	Peritonitis	None	July 1983	Resolved
2	60	F	CAPD	Peritonitis	Cephradine, flucloxacillin, tobramycin	July 1983	Died
3	48	F	Haemodialysis	None	None	July 1983	Remained well
4	15	F	CAPD	Peritonitis	Cephradine, tobramycin	August 1983	Died
5	56	F	Haemodialysis	Wound	Cefuroxime, metronidazole	August 1983	Diarrhoea continued
6	59	F	Haemodialysis	Arteriovenous fistula	Flucloxacillin, benzylpenicillin	August 1983	Resolved
7	61	M	CAPD	Peritonitis	Flucloxacillin, metronidazole, ticarcillin	August 1983	Resolved
8	69	F	CAPD	Peritonitis	Cephradine	September 1983	Resolved
9	59	M	Haemodialysis	Mastoid	Flucloxacillin, benzylpenicillin	October 1983	Resolved
10	50	M	Haemodialysis	Pericolic abscess	Cephradine, cefuroxime, metronidazole	October 1983	Resolved
11	68	F	CAPD	Peritonitis	Tobramycin	November 1983	Resolved
12	71	F	CRF	None	None	January 1984	Resolved
13	73	M	Haemodialysis (acute)	Pneumonia	Ampicillin, cefuroxime, erythromycin, metronidazole, gentamicin, benzylpenicillin	January 1984	Resolved
14	33	F	Haemodialysis	Urinary tract	Co-trimoxazole	February 1984	Resolved
15	63	F	Haemodialysis (acute)	Ischaemic bowel	Cefuroxime, metronidazole, tobramycin	February 1984	Died
16	64	F	CAPD	Peritonitis	Flucloxacillin	February 1984	Resolved
17	60	M	CAPD	Peritonitis	Flucloxacillin	March 1984	Resolved
18	66	F	Haemodialysis	Arteriovenous fistula	Cephradine, cefuroxime, tobramycin	March 1984	Died

CAPD = Continuous ambulatory peritoneal dialysis. CRF = End stage chronic renal failure.